Genes and Mechanisms in Primary Microcephaly

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Introduction

Genes and mechanisms in primary microcephaly

Introduction

Intellectual disability and other neurological diseases in childhood often result from congenital disorders, some being caused by genetic mutations, following Mendelian and non-mendelian (multifactorial) inheritance. Congenital disorders of the brain are sometimes recognizable at birth and affect the structure of the brain and or the head size. One of these disorders is congenital microcephaly, the topic of this thesis. In order to introduce the experimental work I will review the concept and definition, illustrate the importance of studying this topic for the understanding of human brain evolution and present the current hypothesis about disease pathogenesis.

Definition and Prevalence

Microcephaly is defined as a head circumference smaller than 3 standard deviation (SD) when corrected for age and sex. The prevalence of microcephaly, which is difficult to ascertain and is dependent on the threshold used, is estimated to be between 1 and 2.5 % of the adult population (Ulster University data 2003). The prevalence of microcephaly in neurodevelopmental clinic is on average 25%[1].

Microcephaly can be present at birth (primary or congenital) or become evident in the years after birth (acquired or secondary).

Congenital microcephaly has an incidence of approximately 1.6 per 10 000 births (Ulster University data 2003) in the UK. Congenital microcephaly can be further subdivided into syndromic microcephaly and non-syndromic (isolated) microcephaly.

Syndromic microcephaly, i.e. microcephaly with additional features, can be divided based on aetiology into non-genetic and genetic causes.

Non-genetic causes include environmental insults such as exposure to alcohol (foetal alcohol spectrum disorder), radiation, toxins or congenital infections, (e.g. CMV, toxoplasmosis, HIV, rubella) and, rarely, maternal metabolic disorders e.g. maternal PKU.

Syndromic genetic causes of congenital microcephaly can be further subdivided dependent on where the defect lies: numeric or structural chromosomal abnormalities or single gene defects.

Examples of numerical chromosomal abnormalities include trisomy 21 (Down), trisomy 18 and trisomy 13. Examples of structural anomalies detected at routine karyotyping are deletion syndromes; such as 4p or 5p deletion syndrome. Examples of submicroscopic abnormalities detected at microarray analysis are 5q14.3 microdeletion[2] or 1q43q44[3]. In these cases multiple genes are involved in the deletion, but in some cases single genes in the area are considered responsible of the phenotype. The non-chromosomal and monogenetic causes of primary microcephaly are very heterogeneous. A search on the London Medical Database (LMD), to include microcephaly, brings up 758 items. Only a few inborn errors of metabolism are classified as cause of primary microcephaly, such as serine biosynthesis defects and sulphite oxidase/molybdenum cofactor deficiency[4].

Non syndromic microcephaly, as isolated monogenetic microcephaly, is also called “microcephaly vera”. Microcephalia vera is defined by 1) congenital microcephaly of > 3 SD below the age related mean, without craniosynostosis; 2) absence of associated malformations; 3) absence of environmental causes; 4) non-progressive mental retardation (usually mild to moderate), but no other abnormal neurological findings; 5) the presence of seizures does not exclude the diagnosis[5]. This type of primary microcephaly is also indicated as MCPH, and its inheritance is autosomal recessive with nine genes/loci known to be involved until now[6].

The brain morphology in this disorder, as studied at MRI or post-mortem specimens, shows normal or simplified gyration and usually normal or thin brain cortex. The cerebellum can be normal or slightly underdeveloped[7,8,9].

This thesis will concentrate on the monogenetic causes of syndromic primary microcephaly and mechanisms leading to this brain malformation.

Evolution

Cortical size is regulated by a balance between neuroproliferation and apoptosis[10]. Over the last 3 million years a feature of primate evolution has been a 3 times increase in brain size, especially the neocortex[11] and a 100 times increase in gyration along with an increase in cognitive function[12]. As brain size and gyration are correlated with number of neurons it is logical

to look at genes that control proliferation and/ or apoptosis in order to understand the genetic basis of these changes. Therefore the MCPH genes are seen as instrumental to the elucidation of mechanisms regulating brain size and cortical development.

Studies have suggested that the MCPH genes *ASPM* (abnormal spindle-like microcephaly-associated MIM:608176) and *CDK5RAP2* (CDK5 regulatory subunit associated protein 2 MIM:608201) are subject to positive selection for an evolution of increase in brain size over anthropoid primates[13]. *ASPM* is one of the genes most often mutated in patients with primary microcephaly[8,14]. In *ASPM* affected individuals there is a decrease in cortical size but not whole brain or cerebellum size[8]. Philogenetic analysis of *ASPM* and its Drosophila melanogaster homolog abnormal spindle (Asp) have shown the main difference between flies and humans is the large single insertion coding for a so-called "IQ" (isoleucine-glutamine) domain[8]. Gorilla and human lineages show particularly accelerated evolution in this domain[15]. This is consistent with the effect of mitotic spindle on neurogenesis and expression patterns of *ASPM* which is highly expressed in cerebral cortex[8]. Although known primary microcephaly genes are good candidates and are probably contributing factors, the genetic contribution to evolution of cortical size is largely unknown and it is also unclear which selection pressures are involved.

Embryonic Development and neuroproliferation

In order for the brain to develop normally there needs to be rapid and continuous cellular proliferation from the rostral end of the foetal neural tube. A correct balance is required between neural progenitor proliferation and differentiation for normal brain development and brain size[16] which occurs on the ventricular surface. In the ventricular zone the cells undergo symmetric (to produce progenitor cells and expand the progenitor pool) and asymmetrical division (to produce one progenitor and one cell that migrates out of the ventricular zone and differentiates into a neuron). This results in increasing number of neurons and cortical thickness. The balance between symmetrical and asymmetrical division changes over time (initially greater symmetrical division changing to an increased asymmetrical division) in order for the correct amount of neurons to be produced[17]. This is at least partially determined by the plane of the mitotic spindle. Vertical cleavage leads to division of the progenitor cell into two identical progenitor daughter cells, horizontal cleavage results in asymmetric division into an apical and a basal daughter cell to return to the progenitor pool. The other daughter

becomes post- mitotic and differentiates at the pial surface to become neuron (Figure 1)[18].

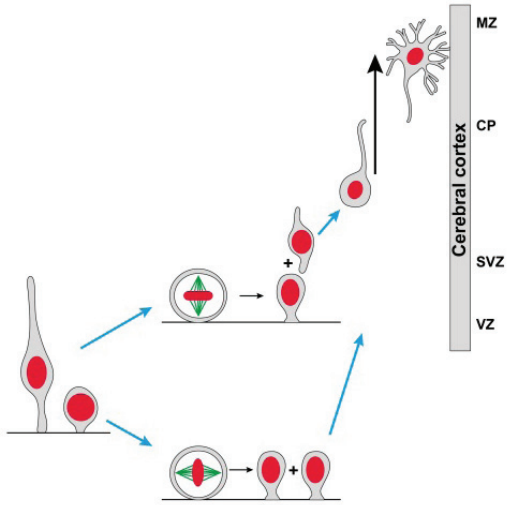

Figure 1. In early cortical development most divisions are in the vertical cleavage plane (lower panel) to give two identical or symmetrical daughter progenitor cell. In later cortical development the cleavage plane changes to become horizontal and give a progenitor and a neuron. This is dependent on the position of the centrosome and mitotic spindle in order to create the correct amounts of neurons and progenitor pool.

The mitotic spindle orientation is directed by the centrosome location. Centrosomes are the main cytoplasmic microtubule organising centre (MTOC) in animal cells; they have a vital role in cell polarity, cell cycle control and cell division. The centrosome is composed of two centrioles surrounded by peri-centriolar material (PCM). During the cell cycle in G1 there are two centrioles in the PCM, at the beginning of the S phase a single pro-centriole forms next to the parent centriole. These two elongate so at the beginning of mitosis two centrosomes are present which anchor microtubules and form the poles of the mitotic spindle and tightly regulate chromosome numbers. Centrosomes are also integral to biosynthesis of cilia as the cilliary basal body is derived from centrioles in order to establish the axoneme. Centrosome abnormalities lead to abnormal mitotic spindle and thus abnormal cell proliferation. Centrosome abnormalities were first linked to cancer as centrosome amplification can lead to chromosome instability, multiple cilia and neuroblast over proliferation[19]. After this discovery, centrosomes were then linked to a variety of human diseases including brain development and ciliopathies[20]. Although the whole process is not completely understood it

is clear that, besides centrosomes, also other mitotic spindle proteins are essential for this process[21]. It has been hypothesized that if this process is disturbed then neuronal proliferation would be decreased and thus cause microcephaly[8,22,23]. In support of this reasoning is that all of the described MCPH genes and some genes that cause Seckel syndrome (a syndromic primary microcephaly) encode for proteins important in the centrosome or mitotic spindle and all are expressed in the neuro-epithelium during embryonic neurogenesis[22,23].

The post mitotic neurons produced by asymmetrical division migrate to their target area by radial migration to form the six layers of the cortex. This occurs with the first neurons making up the innermost layer and later neurons subsequently making up the outer layers to form the cortex in an inside-out fashion[17,24]. Disturbances in this process lead to cortical malformations/migration disorders such as the lissencephalies.

Primary microcephalies present with an apparently normal (or thin) cortex with a decreased number of convolutions, this is usually referred to as simplified gyration. However in primary microcephalies the process of convolution can be abnormal and the small brain can associated with a different sort of malformations such as lissencephaly. This is often referred to as micro-lissencephaly, as seen in biallelic *NDE1* (MIM:614019) mutations[25,26]. In other examples, the microcephaly is associated with abnormal convolution, presenting multiple and shallow gyri with fusion of the gyri, typical of polymicrogyria, as seen in several individuals with *WDR62* (WD repeat-containing protein 62 MIM:613583) mutations[27].

Apoptosis and cortical development

In studies of development of the human neo-cortex it has been found that in the areas of proliferation from week 5 of development[28] there is programmed cell death or “apoptosis” present. Over the next weeks the rate of apoptosis increases and spreads throughout the cortical layers but is highest in the ventricular and subventricular zone[28]. Apoptosis is a complex multi step, multi pathway cell death program which is under complex genetic control, it is the end point of a number of pathways and can be triggered by extra cellular factors such as UV irradiation, or intra-cellular causes such as endoplasmic reticulum (ER) stress, DNA damage and tumour necrosis factor (TNF) pathways[29,30].

The final common pathway in apoptosis from all causes is activation of the caspase cascade. Pro-caspases are cleaved and become active,

these then cleave further pro-caspases in an amplifying reaction and activate further proteins that dismantle the nucleus and DNA and result in death of the cell. Studies in mice show that apoptotic death is essential for determination of the cerebral cortex size[31]. Caspase deficient mice (casp 9 -/-) show hyperplasia of the mouse forebrain. I.e. in animals with a decreased functioning apoptotic pathway the brain size increased[32]. It could therefore be reasoned that mammals with increased apoptosis could have microcephaly. The evidence for deranged apoptotic control causing microcephaly is scarce but has been proposed as a mechanism in several disorders including the DNA repair disorders[33].

Classification of primary Microcephaly

The most widely used classification system for malformations of cortical development, as detected by brain MRI, is designed by James Barkovich and collaborators[34]. Initially in 2001 this was based on a radiological classification[35]. However, due to an increase in knowledge regarding mechanisms and genetic causes, the most recent update is based on a mixture of MRI findings, genetic and clinical information[34]. Congenital microcephaly falls into the “Barkovich’s” group I (within the disorders of neural proliferation or apoptosis) and III (within the disorders of postmigrational development). However, some patients will cross over several groups.

Group I A includes malformations secondary to abnormal neuronal and glial proliferation or apoptosis. This includes the microcephaly with a normal, or thin cortex, and simplified gyration, due to mutations in the MCPH genes and other centrosomal genes that cause the large group of syndromic primordial dwarfism. There are no definable radiological characteristics that can separate out the primary microcephalies. This radiological aspect of this group is often indicated as MSG, i.e. microcephaly with simplified gyration[9].

The mutations in genes involved in migrational development such as *NDE1, TBR2* (T-box brain 2 MIM: 604615) and *WDR62* are also included in the I A group. These mutations, however, lead to severe cortical dysgenesis recognizable as lissencephaly (*NDE1*) or polymicrogyria (*TBR2* and *WDR62*).

Among these three genes only WDR62 mutations can be associated with an apparently normal cortex or simplified gyration[36,37]. Mutations in other genes causing lissencephaly and polymicrogyria, such as *LIS1* (Lissencephaly 1

MIM 601545), and *TUBA1* (tubulin alpha-1-a MIM:611603), can also be associated with borderline microcephaly at birth and secondary stagnation of head growth[38,39]. This latter group is included in the Barkovich's group II B, among the malformations due to generalized abnormal transmantle migration and will not be discussed in detail[34]. Group III D includes malformations with microcephaly secondary to abnormal postmigrational development, with head circumference at -2 SD below the mean at birth and progressing in short time to -4 or more SD This is where brain growth seems to slow after birth because of a rapidly progressing disease process and includes the X-linked *CASK* (calcium dependent-serine-protein-kinase MIM:300749) mutations in females, the pontocerebellar hypoplasia mutations in transfer RNA splicing endonuclease subunit genes *TSEN54* (tRNA splicing endonuclease54 MIM:277470), *TSEN2* (MIM:612389) and *TSEN34* (MIM:612390), and many syndromes defined as developmental encephalopathies such as Rett syndrome caused by *FOXG1* mutation (Forkhead box MIM:613454), Angelman syndrome (MIM:105830):, Pitt-Hopkins syndrome (MIM:610954) and Mowat-Wilson syndrome (MIM:235730) (Table 3).

For the scope of this thesis we will summarise and classify the primary microcephalies according to the radiological and pathological presentation and to the association with other congenital anomalies.

Isolated Primary Microcephaly with normal or thin cortex on MRI (Table 1)

Table 1 summarises all the known primary microcephaly MCPH genes, cellular function, information when available from animal studies and human phenotype. MCPH is defined by the presence of normal or simplified gyration and normal or thin cortex. MCPH refers to autosomal recessive loci identified by linkage analysis in consanguineous pedigrees. A few of them will be discussed in detail as they are relevant to the present work.

The prevalence at birth of MCPH is dependent on population and ranges from 1 per million in the UK to 1 per 10000 in Pakistan reflecting the increased incidence in a consanguineous population due to the AR inheritance pattern[10]. So far 9 loci have been identified and the responsible genes discovered, these are MCPH1 (microcephalin MIM: 251200)*, WDR62, CDK5RAP2, CASK, ASPM, CENPJ* (centromeric protein 7 MIM: 608279)*, STIL* (SCL/TAL-interrupting locus MIM: 18159)*, CEP135*

(centrosomal protein MIM: 611423) and *CEP152* (centrosomal protein MIM: 614852)[22]. It has also become clearer in recent years that MCPH is a genetic and phenotypically heterogenous disorder. The phenotype has particularly been expanded since the discovery of the MCPH2 gene, *WDR62,* by whole exome sequencing[40]. Primary microcephaly is thought to arise from a decrease in neuro-proliferation and/or increase in apoptosis[35]. However, so far none of the known MCPH mutations are known to cause increased apoptosis, and all of the known MCPH genes do appear to encode proteins that have a role in the centrosome and thus probably influence neuroproliferation[41]. Prior to the discovery of *WDR62,* MCPH was associated with most mutations causing a truncated protein and cortical malformations were rare. The brain structure was thought to be preserved with only a simplified gyral pattern being observed and the phenotype of the different MCPHs was thought to be indistinguishable from each other[10]. *ASPM* has been associated with epilepsy, simplified gyral pattern, ventricular enlargement, partial corpus callosum agenesis and cerebellar hypoplasia[43,44]. Besides classic MCPH phenotype, in linkage-unbiased whole exome sequencing studies, *WDR62* mutations have been reported to have a broad clinical phenotype. Patients are reported with cortical malformations such as pachygyria, lissencephaly, schizencephaly and polymicrogyria[36]. The phenotype is not dependent on type of mutation, as chapter 4 explores. *WDR62* and *ASPM* account for over 50% of all cases of MCPH[45]. The function of WDR62 is at present unknown but some investigators have showed that it is a centrosomal protein with an expression pattern similar to ASPM[36, 37].

MCPH1 is a protein that appears to have a dual role in centrosomal organisation and in DNA repair process, corresponding to the three breast cancer susceptibility protein C-terminal (BRCT) domains. The N-terminal BRCT domain has a role in cell cycle regulation and centrosome effects while the tandem BRCT2 and BRCT3 domains have a role in regulation of DNA damage[46]. Loss of MCPH1 leads to a premature entry into mitosis and premature chromosome condensation[47]. MCPH1 is required at multiple points in the DNA damage response including upstream of the ATM/ATR pathway and is required for recruitment of multiple involved proteins. MCPH1 is also indicated in regulation of transcription of DNA damage genes[48]. It is thought that the microcephaly in MCPH1 patients is caused by defective centrosomal function leading to decreased proliferation; however defective DNA repair can also cause microcephaly and this needs further exploration, as DNA repair defects also lead to microcephaly through different mechanisms (see chapter 3).

Syndromic Microcephaly with normal or simplified gyration and a normal or thin cortex (Table 2)

Some of the genes described in MCPH are also involved in Seckel Syndrome and/or microcephalic osteodysplastic primordial dwarfism (MOPD-I, II and III). Seckel and MOPD share microcephaly, intrauterine growth retardation, and distinctive facial features also called “bird headed dwarfism”. Seckel syndrome is a genetically and clinically heterogenous disorder. Known genetic causes include DNA repair genes such as *ATR* (ataxia-telangiectasia and RAD3 related MIM 601215), *RBBP8* (retinoblastoma binding protein-8 MIM 604124) and *NIN* (ninein MIM 614851) as well as the known microcephaly genes including *CENPJ* and *CEP152* (Table 2). At present it is unclear as to why mutations in genes in some patients cause primary microcephaly and others cause Seckel syndrome. However centrosomal proteins are not the complete picture as many genes associated with Seckel syndrome are involved in the ATR-dependent DNA damage signalling pathway[49]. Interestingly, the *Cenpj* hypomorphic mouse, which shows a phenotypic similarity to Seckel syndrome with microcephaly, dwarfism and skeletal abnormalities, has increased apoptosis in the developing telencephalon and a reduced number of neurons which could contribute to the pathogenesis of microcephaly and intellectual disability[50].

Centrosomal protein pericentrin (PCNT MIM:210720) mutations are associated with MOPD type II and Seckel syndrome, again showing an overlap with the MCPH. MOPD (I,II and III) is a disorder characterised by prenatal onset disproportionate short stature with mesomelic limb shortening and other skeletal distinctive features, primary microcephaly and distinctive facies and has a clinical overlap with MCPH and Seckel syndrome[51]. Lack of PCNT leads to disorganisation of the mitotic spindle and missegregation of the centrosome[52]. PCNT is shown to directly interact with MCPH1. A lack of either PCNT or MCPH1 leads to a deficiency of cell cycle check point kinase, CHK1 at the centrosomes, which is essential in regulation of mitosis[53].

Efficient DNA repair is essential for normal formation of the nervous system and microcephaly is a feature of several DNA repair disorders. However, its presence is an inconsistent feature, even within the spectrum of DNA repair syndromes caused by mutations in the same gene such as xeroderma pigmentosum (XP) syndrome and Cockayne Syndrome[54]. The underlying mechanism for congenital microcephaly is unclear in DNA

repair syndromes, although animal models hint to increased cell death being a possible mechanism[55,56]. A recent example of this in humans is discussed in depth in Chapter 3.1 of this thesis. Microcephaly, seizures and developmental delay syndrome (MCSZ, MIM 613402) is an autosomal recessive syndrome described by Shen et al caused by mutations in the polynucleotide kinase 3` phosphatise gene (*PNKP*)[56]. PNKP is involved in repair of both single strand breaks (SSB) and double-strand breaks (DSB)[57,58] Interference studies of Pnkp in mouse neurons showed increased apoptosis in neuronal precursors and in differentiated neurons, suggesting apoptosis to play a role in the pathogenesis of the human disorder[56]. Other severe congenital disorders of DNA repair presenting with microcephaly, such as Cranio-oculo-facial-skeletal (COFS) syndrome are also listed in Table 2. COFS and PNKP-related syndromes are an object of study in Chapter 3.

Examples of primary microcephaly syndromes associated with cerebral and extra cerebral abnormalities (Table 3)

Primary microcephaly can be associated with cerebral and extra cerebral abnormalities. We have summarised in Table 3 most syndromes consistently associated with head circumference below 3SD at birth. This list includes the pontocerebellar hypoplasia`s (PCH) sub types 1-7 which present with hypoplasia of the cerebellum and the pons, progressive microcephaly, and variable cortical features, mostly simplified gyration or generalised hypoplasia. *PCH2* (MIM:277470), *PCH4* (MIM:225753) and *PCH5* (MIM: 610204) are associated with mutations in three tRNA subunit endonuclease subunits involved in tRNA splicing[59].

Wolcott-Rallison syndrome (WRS, MIM 226980) is an example of a multi-system syndrome which presents with neonatal diabetes, bony abnormalities and microcephaly with a simplified gyral pattern and intractable seizures[60]. WRS is caused by *EIF2AK3* mutations; this gene is involved in the regulation of protein synthesis during cellular stress conditions. The causative mechanism is increased apoptosis secondary to endoplasmic reticulum stress to which neurons and Islet of Langerhans cells seem to be particularly susceptible[61,62]. In this thesis patients with a phenotypic similarity to WRS, who do not bear the *EIF2AK3* mutation as described leading to the hypothesis that this separate syndrome, also had increased susceptibility to apoptosis as a mechanism for the microcephaly and diabetes (chapter 2).

Lissencephaly is defined as smooth and thickened cerebral cortex with a sparse cell zone caused by defective neuronal migration during midgestation. Barkovich's classification of cortical malformations includes a category of microcephaly with lissencephaly[34]. The identified genetic defects causing lissencephaly are *LIS1*, *DCX* (doublecortin MIM: 300121), *RELN* (reelin MIM: 600514), and *TUBA1A*. In this spectrum there are reports of association of microcephaly and lissencephaly known as "microlissencephaly" (MLIS) but, in general, microcephaly is not a consistent feature of lissencephaly[38]. *NDE1* mutations cause LIS4 or microlissencephaly 4. Extreme microcephaly is a consistent LIS4 feature with the head circumference of affected individuals -10 SD below expected[26]. NDE1 interacts with LIS1 and DCX participating in the microtubule organising centre for nucleokinesis during neuronal migration, both proteins being encoded by genes involved in classic lissencephaly[63]. NDE1 protein has been shown to have a role in mitotic spindle formation and neuronal proliferation[25] showing similarities to the MCPH proteins. Interestingly *WDR62* (MCPH2) mutations can be associated with a range of cortical malformations (such as pachygyria and polymicrogyria), or only a simplified gyration[27,36,37,64,65,68]. This raises the question whether other (genetic) factors contribute to this phenotypic variability.

In summary, what has previously been thought to be entirely separate conditions with distinct phenotype and genotypes have been shown in recent years to be more of a continuum with interaction and overlap between centrosomal proteins and DNA repair pathways.

Practical Diagnostic approach to a child with microcephaly

A flowchart may be of use to the clinician who is confronted with a microcephalic child in order to accurately diagnose the type of microcephaly and to decide on relevant further investigations (figure 2). A thorough family, prenatal and birth history, and a complete examination are essential, and should include ophthalmological and hearing screening.

If there are specific additional features suggesting a syndromic cause, ophthalmological assessment is especially vital. This can guide to specific, or even pathognomonic, signs of syndromal or infectious causes. In 48% of syndromes with microcephaly there is also ophthalmological involvement which can help diagnosis[1].

Neuroimaging, preferably MRI, in children with a head circumference of greater then -3SD below the mean has a diagnostic yield of between

58-80%[67, 68]. Also, there is a correlation between brain abnormalities and developmental abilities on the Bayley scales of development[69].

Although microcephaly is a genetically and clinically heterogenous disorder with phenotypical overlap, neuroimaging can guide us to targeted genetic testing and also has a prognostic value. Figure 2 summarises a diagnostic approach to the child with congenital microcephaly.

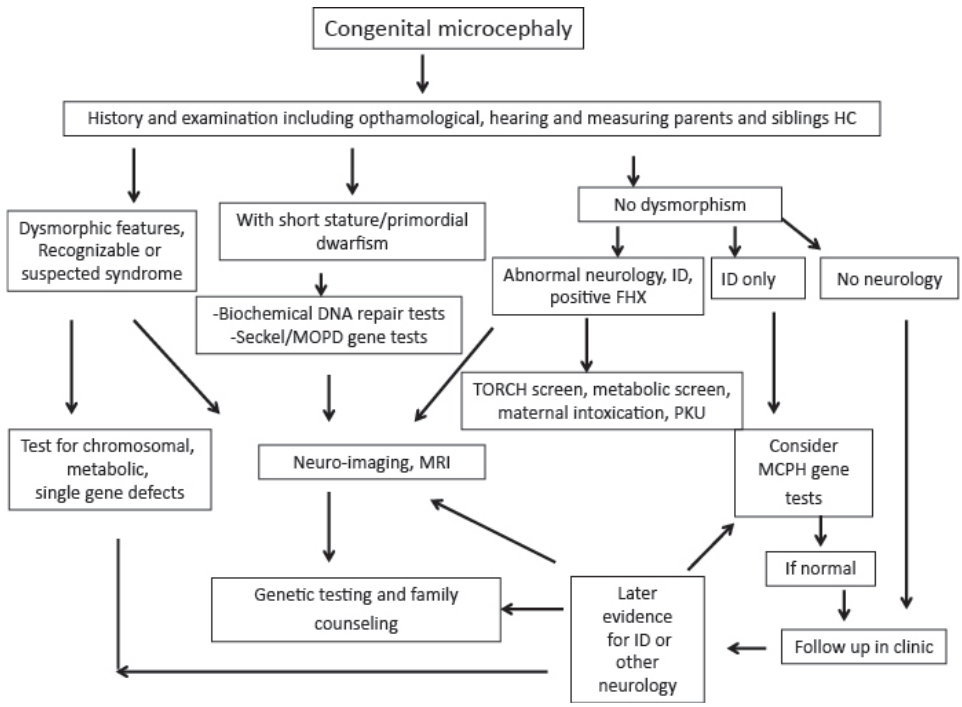

Figure 2. Flow diagram summarising a clinical approach including investigations to a child with congenital microcephaly.

Scope of the Thesis

Genetic studies of causes of congenital microcephaly have mostly been focused on disorders of proliferation. Even though in Barkovichs classification it states that “increase in apoptosis of neuroprogenitors” can be a causative mechanism[34], at the beginning of this study no forms of microcephaly in humans had been attributed to this mechanism. The aim

of this thesis is to describe new syndromes associated with microcephaly, novel genetic mutations and phenotypic variations, making use of state of the art genomic technologies. Additionally we provide some insights to novel mechanisms involving primary dysregulation of apoptosis of neuroprogenitors.

In **Chapter 2 (Brain Development and apoptosis)** we applied traditional linkage analysis to data from SNP-arrays in order to identify the genetic cause of a new syndromic primary microcephaly. Two families are presented with a combination of microcephaly with simplified gyration (MSG) and persistent neonatal insulin-dependent diabetes caused by a mutation in *IER3IP1*. Disease mechanism is attributable to increased apoptosis of neuronal progenitor cells and pancreatic beta islet cells, which was observed with studies on patient fibroblasts and post-mortem material.

In **Chapter 3 (DNA repair and microcephaly)** we approach the phenotypes and pathogenesis of microcephaly in DNA repair disorders.

In **Chapter 3.1** we applied whole genome sequencing (WGS) to identify the genetic mutation in a yet undefined syndromic microcephaly associated with progressive cerebellar atrophy, ataxia and severe peripheral polyneuropathy. We describe the phenotypic expansion of a known DNA repair syndrome caused by PNKP mutations in this family. We can explain the presentation by looking at predicted protein interactions and confirm abnormal control of apoptosis as the proposed pathogenic mechanism.

In **Chapter 3.2** severe cases of cerebro-oculo-facio-skeletal (COFS) syndrome caused by *ERCC5* mutations who presented with a severe congenital microcephaly are described. Apoptosis is apparently implicated in the pathogenic mechanism of microcephaly in COFS with evidence from patient fibroblasts and mouse studies. We applied functional studies in human material to support our hypothesis.

In **Chapter 4 (Microcephaly and cortical malformations),** we tested the hypothesis that the phenotypic spectrum of cortical malformation in microcephaly can result from the contribution of multiple factors. Individuals with *WDR62* mutations and variable phenotypic abnormalities are described. The observation of a mutation in *TBCD*, the gene for tubulin cofactor D, in the most severely affected individual leads to a proposal of polygenic inheritance as the hypothesis for explaining the wide phenotypic variation in *WDR62* patients.

References

1. Ashwal S, Michelson D, Plawner L, and Dobyns W.B (2009) Practice Parameter: Evaluation of the child with microcephaly (an evidence-based review) Neurology 73:887-897.

2. Shimojima K, Okumura A, Mori H, Abe S, Ikeno M, Shimizu T, Yamamoto T (2012) De novo microdeletion of 5q14.3 excluding MEF2C in a patient with infantile spasms, microcephaly, and agenesis of the corpus callosum. Am J Med Genet Part A 158A:2272-2276.

3. Ballif BC, Rosenfeld JA, Traylor R, Theisen A, Bader PI, Ladda RL, Sell SL, Steinraths M et al (2012) High-resolution array CGH defines critical regions and candidate genes for microcephaly, abnormalities of the corpus callosum, and seizure phenotypes in patients with microdeletions of 1q43q4.2012. Hum Gen 131:1:145-56.

4. Saudubray JM, van den Berghe G, Walter JH (2012) Inborn Metabolic Diseases:Diagnosis and Treatment. 5th Edition. Springer.

5. Jackson AP, McHale DP, Campbell DA, Jafri H, Rashid Y, Mannan J, Karbani G, Corry P et al (1998) Primary autosomal recessive microcephaly (MCPH1) maps to chromosome 8p22-pter.Am J Hum Genet 63:541-546.

6. Mahmood S, Ahmad W, Muhammad H. (2012) Autosomal recessive primary microcephaly (MCPH): clinical manifestations, genetic, heterogeneity and mutation continuum.Orphanet Journal of Rare Diseases 6:39.

7. Barkovich AJ, Kuzniecky RI, Jackson GD, Guerrini R, Dobyns WB (2005) A developmental and genetic classification for malformations of cortical development. Neurology 65:1873-1887.

8. Bond J, Roberts E, Mochida GH, Hampshire DJ, Scott S (2002) ASPM is a major determinant of cerebral cortical size. Nat Genet 32:316-320.

9. Tarrat A, Garel C, Germanaud D, de Villemeur TB, Mignot C, Lenoir M, le Pointe HD (2009) Microcephaly: a radiological review. Pediatr Radiol 39:888-9.

10. Woods CG, Bond J, Enard W (2005) Autosomal recessive primary microcephaly (MCPH): a review of clinical, molecular, and evolutionary findings. Am J Hu. Genet 76:717-28.

11. Clark DA, Mitra PP, Wang SS (2001) Scalable architecture in mammalian brain. Nature 411:189-193.

12. Rakic P (1995) A small step for cell, a giant leap for mankind: a hypothesis of neocortical expansion during evolution. Trends Neurosci 18:383-8.

13. Montgomery SH, Capellini I, Venditti C, Barton RA, Mundy NI (2011) Adaptive Evolution of four microcephaly genes and evolution of brain size in Anthropoid primates. Mol.Biol.Evol 28:625-38.

14. Darvish H, Esmaeeli-Nieh S, Monajemi G.B, Mohseni M, Ghasemi-Firouzabadi S, Abedini S.S, Bahman I, Jamali P, Azimi S, Mojahedi F et al (2010) A clinical and molecular genetic study of 112 Iranian families with primary microcephaly. J Med Genet 47:823-828.

15. Kouprina N, Pavlicek A, Mochida GH, Solomon G, Gersch W, Yoon YH, Collura R, Ruvolo M, Barrett JC, Woods CG, Walsh CA, Jurka J, Larionov V (2004) Accelerated evolution of the ASPM gene controlling brain size begins prior to human brain expansion. PLoS Biol 2:E126.

16. Chenn A, Walsh CA (2002) Regulation of cerebral cortical size by control of cell cycle exit in neural precursors. Science 297:365-369.

17. Noctor SC, Martinez-Cerdeno V, Kriegstein A.R (2004) Cortical neurons arise in symmetric and asymmetric division zones and migrate through specific phases. Nat Neurosci 7:136-44.

18. Chenn A, McConnell SK (1995) Cleavage orientation and the asymmetric inheritance of Notch1 immunoreactivity in mammalian neurogenesis. Cell 82:631-41.

19. Nigg EA, Raff JW (2009) Centrioles, centrosomes and cilia in health and disease. Cell 139:663-78.

20. Bettencourt-Dias M, Hildebrabdt F, Pellman D, Woods G, Godhino S.A (2011) Centrosomes and cilia in human disease.Trends Genet 27:307-315.

21. Kosodo Y, Roper K, Haubensak W, Marzesco A.M, Corbeil D, Huttner W.B (2004) Asymmetric distribution of the apical plasma membrane during neurogenic divisions of mammalian neuroepithelial cells. EMBO J 2:2314-24.

22. Thornton GK, Woods CG (2009) Primary microcephaly: do all roads lead to Rome? Trends Genet.25:501-10.

23. Manzini MC, Walsh CA (2011) What disorders of cortical development tell us about the cortex:one plus one does not always make two. Curr Opin Genet Dev 21:333-9.

24. Rakic P, Ayoub AE, Breunig JJ, Dominguez MH (2009) Decision by division: making cortical maps. Trends Neurosci 32:291-301.

25. Alkuraya FS, Cai X, Emery C, Mochida GH, Al-Dosari MS, Felie JM, Hill RS, Barry BJ *et al* (2011) Human Mutations in *NDE1* Cause Extreme Microcephaly with Lissencephaly. Am J Hum Genet 88: 536-547.

26. Bakiricioglu M, Carvalho OP, Khurshid M, Cox JJ, Tuysuz B, Barak T, Yilmaz S, Caglavan O *et al* (2011) The Essential Role of Centrosomal NDE1 in Human Cerebral Cortex Neurogenesis. Am J Hum Genet 88:523-35.

27. Murdock DR, Clark GD, Bainbridge MN, Newsham I, Wu YQ, Muzney DM, Cheung SW, Gibbs RA, Ramocki MB (2011) Whole-Exome Sequencing Identifies Compound Heterozygous Mutations in *WDR62* in Siblings with Recurrent Polymicrogyria. Am J Med Gen 155A(9):2071-7.

28. Chan WY, Lorke DE, Tiu SC, Yew DT (2002) Proliferation and apoptosis in the developing human neocortex *et al.* Anat Rec. 1:267:261-76.

29. Merksamer PI,Papa FR (2010) The UPR and cell fate at a glance J. Cell Sci. 123:1003-1006.

30. Zimmermann KC, Green DR (2001) How cells die: apoptosis pathways. J Allergy Clin Immunol 108:s99-103.

31. Hayder TF, Kuan CY, Flavell RA, Rakic P (1999) The role of cell death in regulating the size and shape of the mammalian forebrain. Cereb Cortex 9:621-6.

32. Kuida K, Haydar TF, Kuan CY, Gu Y, Taya C, Karasuyama H, Su MS, Rakic P, Flavell RA (1998) Reduced apoptosis and cytochrome c-mediated caspase activation in mice lacking caspase 9. Cell 94: 325-337.

33. Soltys D, Rocha CRR, Lerner LK, de Souza TA, Munford V, Cabral F, Nardo T, Stefanini M, Sarasin A, Cabral-Neto JB, Menck CFM (2013) Novel XPG (*ERCC5*) mutations affect DNA repair and cell survival after ultraviolet but not oxidative stress. Hum Mut 34:348-489.

34. Barkovich AJ, Guerrini R, Kuzniecky RI, Jackson GD, Dobyns WB (2012) A developmental and genetic classification for malformations of cortical development: update 2012. Brain 135:1348-69.

35. Barkovich A.J, Kuzniecky R.I, Jackson G.D, Guerrini R, Dobyns W.B (2001) Classification system for malformations of cortical development update 2001. Neurology 57:2168-78.

36. Yu TW, Mochida GH, Tischfield DJ, Sgaier SK, Flores-Sarnat L, Sergi CM, Topçu M, McDonald MT, Barry BJ et al (2010) Mutations in *WDR62*, encoding a centrosome-associated protein, cause microcephaly with simplified gyri and abnormal cortical architecture. Nat Genet 42:11.

37. Nicholaas A.K, Khurshid M, Desir J, Carvalho OP, Cox JJ, Thornton G, Kausar R, Ansar M, Ahmad W et al (2010) WDR62 is associated with the spindle pole and is mutated in human microcephaly. Nature Genetics 42:1010-1015.

38. Sohal A.P, Montgomery T, Mitra D, Ramesh V (2012) *TUBA1A* Mutation-Associated Lissencephaly: Case Report and Review of the Literature. Pediatr Neurol 46:127-131.

39. de Wit M.C, de Rijk-van Andel J, Halley DJ, Poddighe PJ, Arts WF, de Coo IF, Mancini GM (2011) Long-term follow-up of type 1 lissencephaly: survival is relayed to neuroimaging abnormalities. Dev Med Child Neurol 53:417-21.

40. Bilgüvar K, Oztürk AK, Louvi A, Kwan KY, Choi M, Tatli B, Yalnizoğlu D, Tüysüz B, Cağlayan AO, et al (2010) Whole exome sequencing identifies recessive *WDR62* mutations in severe brain malformations. Nature 9:201-210.

41. Kaindl A.M, Passemard S, Kumar P, Kraemer N, Issa L, Zirner A, Gerard B, Verloes A, Mani S, Gressens P (2010) Many roads lead to primary autosomal recessive Microcephaly. Prog Neurobiol 90:3: 363-366.

42. Wollnik A (2010) A common mechanism for microcephaly. Nat Genet 42:11:923-924.

43. Shen J, Eyaid W, Mochida GH, Al-Moayyad F, Bodell A, Woods C G, Walsh CA (2005) *ASPM* mutation identified in patients with primary microcephaly and seizures. J Med Genet 42: 725-729.

44. Passemard S, Titomanlio L, Elmaleh M, Afenjar A, Alessandri JL, Andria G, Billette de Villemeur T, Boespflug-Tanguy,O, Burglen L, Del Giudice E, Guimiot F, Hyon C et al (2009) Expanding the clinical and neuroradiologic phenotype of primary microcephaly due to *ASPM* mutations. Neurology 73: 962-969.

45. Hussain S, Marriam Bakhtiar M, Farooq S, Anjum I, Janzen E, Toilat M, Eiberg H, Kjaer K, Tommerup N *et al* (2012) Genetic heterogeneity in Pakistani microcephaly families. Clin Gen: 1-6.

46. Lin SY, Rai R, Li K, Xu ZX, Elledge S J (2005) BRIT1/MCPH1 is a DNA damage responsive protein that regulates the Brca1-Chk1 pathway, implicating checkpoint dysfunction in microcephaly. Proc Nat Acad Sci 102: 15105-15109.

47. Alderton GK, Galbiati L, Griffith E, Surinya K H, Neitzel H, Jackson AP, Jeggo PA, O'Driscoll M (2006) Regulation of mitotic entry by microcephalin and its overlap with ATR signalling. Nature Cell Biol. 8: 725-733.

48. Xu X, Lee J, Stern D F (2004) Microcephalin is a DNA damage response protein involved in regulation of CHK1 and BRCA1. J. Biol. Chem 279: 34091-34094.

49. O'Driscoll M, Ruiz-Perez VL, Woods CG, Jeggo PA, Goodship JAA (2003) Splicing mutation affecting expression of ataxia-telangiectasia and Rad3-related protein (ATR) results in Seckel syndrome. Nature Genet. 33: 497-501.

50. McIntyre RE, Lakshminarasimhan Chavali P, Ismail O, Carragher DM, Sanchez-Andrade G, Forment JV, Fu B, Del Castillo Velasco-Herrera M et al (2012) Disruption of mouse Cenpj, a regulator of centriole biogenesis phenocopies Seckel Syndrome. PLos Genetics. 8:11 1-18.

51. Klingsieson A, Jackson AP (2011) Mechanisms and pathways of growth failure in primordial dwarfism. Genes Dev 25:2011-2024.

52. Rauch A, Thiel C.T, Schindler D, Wick U, Crow Y.J, Ekici A.B, van Essen A.J, Goecke T.O et al (2008) Mutations in pericentrin (*PCNT*) gene cause primordial dwarfism. Science 8: 319:816-9.

53. Tibelius A, Marhold J, Zentgraf H, Heilig CE, Neitzel H, Ducommun B, Rauch A, Ho AD et al (2009) Microcephalin and pericentrin regulate mitotic entry via centrosome associated Chk1. J Cell Biol 185: 1149-1157.

54. Subba Rao K (2007) Mechanisms of Disease: DNA repair defects and neurological disease. Nat Clin Prac Neurology 3:162-171.

55. Gilmore EC, Nowakowski RS, Caviness VS Jr, Herrup K (2000) Cell birth, Cell death, Cell diversity and DNA breaks: how do they all fit together. Trends Neurosci 23:91-140.

56. Shen J, Gilmore EG, Marshall CA, Haddadin M, Reynolds JJ, Eyaid W ,Barry B, Gleason D, Allen K, Ganesh VS, Chang BS, A Grix, Hill RS, Topcu M, Caldecott KW, Barkovich AJ, Walsh CA (2010) Mutations in *PNKP* cause microcephaly, seizures and defects in DNA repair. Nat Genet:42;24: 5-9.

57. Caldecott KW (2008) Single strand break repair and genetic disease. Nat Rev Genet. 9:619-631.

58. Weinfeld M, Mani RS, Abdou I, Aceytuno RD, Glover JN (2011) Tidying up loose ends: role of polynucleotide kinase/phosphatise in DNA strand break repair. Trends Biochem Sci 36:262-71.

59. Namavar Barth PG, Poll-The BT, Baas F (2011) Classification, diagnosis and potential in Pontocerebellar hypoplasia. Orphanet J Rare Dis. 6:50.

60. de Wit MCY, de Coo IFM, Julier C, Delephine M, Lequin MH, van de Laar M ,Sibbles BJ Bruining GJ, Mancini, GMS (2006) Microcephaly and simplified gyral pattern of the brain associated with early onset insulin-dependent diabetes mellitus. Neurogenetics 7:259-63.

61. Harding HP, Zhang Y, Bertolotti A, Zeng H, Ron D (2000) Perk is essential for translational regulation and cell survival during the unfolded protein response. Mol Cell 5:897-904.

62. Harding HP, Zeng H, Zhang Y, Jungries R, Chung P, Plesken H, Sabatini DD,Ron D (2001) Diabetes mellitus and exocrine pancreatic dysfunction in perk-/- mice reveals a role for translational control in secretory cell survival. Mol. Cell 7:1153-1163.

63. Feng Y, Olson EC, Stukenberg PT, Flanagan LA, Kirschner MW, Walsh CA (2000) LIS1 regulates CNS lamination by interacting with mNudE, a central component of the centrosome. Neuron 28:3: 665-79.

64. Bacino CA, Luis AA, Wiszniewska J, Bonnen PE (2011) WDR62 missense mutation in a Consanguinous Family with Primary Microcephaly. Am J Med Gen:158A:622-625.

65. Kousar R, Jawad Hussan M, Khan B, I Basit S, Mahmood S, Mir A, Ahmad W, Ansar M (2011) Mutations in *WDR62* gene in Pakistani families with autosomal recessive primary microcephaly. BMC Neurology:11:119.

66. Memon M.M, Raza S.L, Basit S, Kousar R, Ahmad W, Ansar M (2013) A novel *WDR62* mutation causes primary microcephaly in a Pakastani family. Mol Biol Rep .40:1:591-5.

67. Steinlin M, Zürrer M, Martin E, Boesch C, Largo RH, Boltshauser E (1991) Contribution of magnetic resonance imaging in the evaluation of microcephaly. Neuropediatrics 22:184-189.

68. Sugimoto T, Yasuhara A, Nishida N, Murakami K, Woo M, Kobayashi Y (1993) MRI of the head in the evaluation of Microcephaly. Neuropediatrics 24:4-7.

69. Custer DA, Vezina LG, Vaught DR, Brasseux C, Samango-Sprouse CA, Cohen MS, Rosenbaum KN (2000) Neurodevelopmental and Neuroimaging correlates In nonsyndromal microcephalic children. J Dev Behav Pediatr 21:12-1.

Table 1. Isolated Primary Microcephaly with normal or thin cortex on MRI.

Disease	MIM	Inher-itance	Gene	Protein	Cellular function	Human Phenotype	Animal Models
MCPH1	251200	AR	MCPH1	Micro-cephalin	Located in nucleus, role in DNA damage repair and chromosome condensation	Primary microcephaly, short stature	Drosophila/mice mcph1 -/- reduced microcephalin in centrosomes and abnormal spindles, normal brain size
MCPH2	**604317**	**AR**	***WDR62***	**WDR62**	**Unknown but localises to centrosome and nucleus in similar pattern to ASPM**	**Variable; includes microcephaly, lissencephaly, schizencephaly, polymicrogyria**	
MCPH3	604804	AR	CD-K5RAP2	CDK5 RAP2	Centrosomal protein, regulation of microtubule function and centrosome maturation	Primary microcephaly	Drosophila asp-/- centrosome dysfunction, subtle defect of asymmetric division
MCPH4	609173	AR	*CASC5*	CASC5	2 crucial functions during mitosis, essential for spindle-assemble checkpoint and kinetocore formation.	Primary microcephaly	
MCPH5	605481	AR	*ASPM*	ASPM	Concentrated at mitotic spindle poles and regulates spindle orientation during embryonic neurogenesis	MSG, epilepsy has been reported, enlarged ventricles, partial agenesis of corpus callosum, cortical dysplasia	In Drosophila aspm-/- mitotic arrest. Mice Aspm -/- defects in spindle orientation, reduced brain size

MCPH6/ SECKL4	608393/ /613676	AR	*CENPJ*	CENPJ	Centrosomal protein during mitosis and mitotis spindle poles during prometaphase and metaphase	Microcephaly Seckel Syndrome: Microcephaly, mental retardation, intra-uterine growth retardation, post natal dwarfism, bird like face.	SAS-4 -/- adult Drosophila have no cilia or flagella Mice Cenpj tm/tm dwarfism, microcephaly, memory impairment, skeletal impairment
MCPH7	612703	AR	*STIL*	STIL	Pericentrosomal, spindle organisation, cell cycle progression	Microcephaly, short stature, strabismus, ataxia, seizures	Mouse KO embryonically lethal, failure of neural tube closure, holoprosencephaly, block in shh. Increased apoptosis
MCPH8	614673	AR	*CEP153*	CEP153	Centrosomal protein	Primary microcephaly Severe cognitive defects and unintelligible speech	
MCPH9/ SCKL5	613	AR	*CEP152*	CEP152	Core protein of centrosome, involved in centriole duplication	Primary microcephaly Seckel Syndrome (see above)	Drosophilia mecD mutant characteristic of a ciliary defect

Table 2. Syndromic Microcephaly with normal or simplified gyration and a normal or thin cortex.

Disease	MIM	Inher-itance	Gene	Protein	Cellular function	Human Phenotype	Animal Models
COFS4/CS	610758	AR	ERCC1	ERCC1	DNA repair protein associates with XPA and ERCC4, role in damage recognition and incision activities	Cerebro-oculo-facial-skeletal (COFS)syndrome: microcephaly, micrognathia, severe intellectual disability, distinctive facial features	Homozygous muted mice runted at birth died before weaning. ERCC1 deficient mice growth arrest in 2nd week, death by 4th week
COFS2, Tri-chothiod-ystrophy/ XPD	610756 /601675 /278730	AR	ERCC2	XPD	DNA repair implicated in UV damage, and nucleotide excision repair	COFS syndrome	Mice ERCC2 mutation arg722 to trp symptoms of premature ageing
COFS3/ XPG	**278780**	**AR**	ERCC5	ERCC5	Required for 3`incision during DNA nucleotide excision repair	COFS syndrome	**Mice with mutations in c-terminal deletions have growth retardation. Also see chapter 3b**
COFS/CS type B	214150/ 133540	AR	*ERCC6*	ERCC6	Part of nucleotide excision pathway	COFS and Cockayne syndrome (CS)	
Micro-cephaly, seizures and devel-opmental delay	**613402**	**AR**	***PNKP***	**PNKP**	**Role DNA repair**	**Microcephaly, seizures and developmental delay, also cerebellar atrophy and ataxia + polyneuropathy**	

Nijmegan Breakage syndrome	251260	AR	*NBS1*	NBS1	Repair of double strand breaks (DSBs) forms a complex with hMRE11/ RAD50	Microcephaly, growth retardation, immunodeficiency and predisposition to cancer	
MCPHA (Amish lethal microcephaly)	607196	AR	SLC 25A19	?	Mutations cause a thiamine deficiency, causes a deficient activity of pyruvate dehydrogenase and alpha-ketoglutaric acid dehyrogenase	Severe microcephaly, profoundly delayed psychomotor development, episodic encephalopathy, early lethal.	Slc25a19 knockout mice: 100% prenatal lethality, neural tube closure defects
SCKL1	210600	AR	ATR	ATR	Essential component of DNA damage checkpoint pathway Role in centrosome instability	Seckel Syndrome: severe short stature, microcephaly, and developmental delay Familial Cutaneous Telangiectasia	ATR-/- mice incompatible with life ATR Seckel mice microcephaly, facial dysmorphism, accelerated aging
SCKL2	606744	AR	*RBBP8*	RBBP8	It is also associated with BRCA1 and is thought to modulate the functions of BRCA1 in transcriptional regulation, DNA repair, and/or cell cycle checkpoint control.	Seckel Syndrome Jawad Syndrome: microcephaly, intellectual disability, and digital anomalies	
SCKL3	608644	AR	*unknown*	-		Seckel Syndrome	
SCKL4 and SCKL5 (see table 1)							

SCKL6	614728	AR	*CEP63*	CEP63	subunit of the centrosome, core centriole assembly factor	Seckel Syndrome	
SCKL7		AR	*NIN*	NIN	Centrosomal protein required for centrosome to act as a microtubule organising centre	Seckel Syndrome	Zebrafish morpholino knock down resulting in neuro-ectodermal abnormalities with similarities to human phenotype
MOPD II	210720	AR	*PCNT*	PCNT	Essential for mitotic spindle organisation and missegregation of chromosomes	MOPD II: Microcephaly, microdontia , delayed development, skeletal problems, delayed bone age	
MOPD I-III/TALS	210710	AR	U4atac	none	U4 snRNA component of the minor spliceosome	MOPD I and III and Taybi-Linder cephaloskeletal dysplasia (TALS) are apparently allelic disorders. Some patients have pachygyria, polymicrogyria, callosal hypoplasia and migration defects at autopsy	
Bowen-Conradi	211180	AR	*EMG1*	EMG1	Processing of 18srRNA and small subunit ribosomal assembly and play role in methylation during ribosome biogenesis	Bowen-Conradi syndrome: microcephaly, micrognathia, IUGR, joint limitation	

Table 3. Examples of primary microcephaly syndromes associated with cerebral and extracerebral abnormalities.

Disease	MIM	Inheritance	Gene	Protein	Cellular function	Human Phenotype	Animal Models
Mental Retardation + Microcepaly + Pontocerebellar hypoplasia (MICPCH)	300749	X-linked	*CASK*	CASK	Member of membrane associated guanide kinase (MAGUK) family cytoskeletal membrane scaffold, co-ordinates signal transduction in cortical cytoskeleton	MICPCH ID +/- nystagmus simplified gyral pattern and pontocerebellar hypoplasia; mostly females	CASK -/- mice die soon after birth, partially penetrant cleft palate, apoptosis in thalamus
Pontocerebellar hypoplasia 2a and 4	277470 225753	AR	*TSEN54*	TSEN54	Functions in multiple RNA processing event	PCH 2a/4:microcephaly, extreme cerebellar and pons hypoplasia compared to brain; “bat wing”anomaly at MRI; dysphagia, clonus, impaired vision, seizures,	
Cerebellar hypoplasia and ID +/- quadrupedal locomotion 1	224050	AR	*VLDLR*	VLDLR	Encodes very low density lipoprotein receptor. Reelin acts via VLDLR to regulate microtubule function in neurons	congenital cerebellar ataxia and ID, inferior cerebellar hypoplasia and mild gyral simplification	
Haemorrhagic destruction of brain, subependymal calcification and cataracts	613730	AR	*JAM3*	JAM3	Member of Junctional Adhesion Molecule family, Ig like molecule essential for maintaining integrity of the cerebrovascular endothelium.	Haemorrhagic destruction of brain, subependymal calcification, congenital cataracts, die in early infancy, or survive with profound developmental delay, spasticity and seizures	

Warburg micro Syndrome 1	600118	AR	*RAB-3GAP1*	RAB-3GAP1	Implicated in regulation of exocytosis of neurotransmitters and hormones	Ocular and neurodevelopmental defects and hypothalamic hypogenitalism; microcephaly with polymicrogyric cortex	
Martsolf Syndrome/ Warburg micro syndrome 2	212720 614225	AR	*RAB-3GAP2*	RAB-3GAP2	Implicated in regulation of exocytosis of neurotransmitters and hormones	Microcephaly, polymicrogyria, ID, cataracts, hypogonadism, and short stature	
Warburg micro syndrome 3	614222	AR	*RAB18*	RAB18	Regulate membrane trafficking in organelles and transport vesicles	Microcephaly, ocular and neurodevelopmental disability and hypogenitalism	Zebrafish morphants: microphthalmia, microcephaly, pericardial oedema, delayed jaw formation, overall reduced size and developmental delay
Cohen Syndrome	216550	AR	*COH1*	VPI3B	Potential transmembrane protein may function in vesicle mediated transport and sorting of proteins within a cell	Presentation highly variable, microcephaly, developmental delay, distinctive facial features, truncal obesity, overly social behaviour, joint hypermobility, high myopia/ or retinal dystrophy, neutropenia	
Hoyeraal-Hreidarsson syndrome	300240	AR	*DKC1*	Dyskerin	Small nucleolar ribonucleoprotein particle that modify specific uridine residues of ribosomal RNA. Is also a centromere or microtubule protein	Microcephaly, IUGR, ID cerebellar malformation, pancytopenia, mutations can also cause dyskeratosis congenita	Dkc1 mice with large deletion 100% embryonic lethality from maternal origin, paternally derived deletion in female mice – skewed x –inactivation

Wolcott-Rallison Syndrome	**226980**	**AR**	***EIF2AK3***	**eIF2-alpha**	**Mediates response to malfolded proteins to inhibit translation**	**Neonatal diabetes, spondylo-epiphyseal dysplasia and microcephaly with simplified gyral pattern, seizures**	**Perk -/- mice islets of Langerhan degenerated, exocrine pancreas apoptosis after 4th week, osteoporosis**
Microcephaly, epilepsy and diabetes	**614231**	**AR**	***IER3IP1***	**IER3IP1**	**Localises to Endoplasmic Reticulum, role in apoptosis?**	**Microcephaly with simplified gyral pattern, intractable seizures, neonatal diabetes**	
Bohring-Opitz syndrome	605039	AR/ *de novo* AD?	*ASXL1*	ASXL1	Probable polycomb protein involved in transcription regulation	IUGR, FTT, facial dysmorpism, microcephaly with prominent metopic suture, exopthalmos, facial hemangioma, hirsutism	
Microcephaly +/- chori-oretinopathy, lymphedema, or ID	152950	AD	*KIF11*	KIF11	Plus ended directed microtubule motor functions during mitosis	Microcephaly, +/- chorioretinopathy, dysthichiasis, lymphedema or ID	
Lissencephaly 3	611603	AD	*TUBA1A*	TUBA1A	Encodes for main component of microtubules	Congenital microcephaly, ID, dysgenesis of corpus callosum, cerebellar band hypoplasia, heterotopias, lissencephaly	S140G mice mutants: impaired neuronal migration, impaired special memory, reduced anxiety, abnormal nesting
Microlissen-cephaly (Lis4)	614019	AR	*NDE1*	NDE1	Role in mitotic spindle organisation, neuronal proliferation and cerebral cortical neurogenesis	Lissencephaly, extreme microcephaly, profound ID	Nde1 null mice viable, small brain predominantly the cerebral cortex

Microcephaly with other structural brain abnormalities	-	AD	*TUBB5*	TUBB5; tubulin beta-5	Highly expressed in developing cortex	Microcephaly (-2.5/-4SD) with dysmorphic basal ganglia, corpus callosum dysgenesis, brain stem hypoplasia, focal polymicrogyria	Perturbation of cell cycle in neuronal progenitors and altered position of migrating neurons
Microcephaly-Capillary-Malformation-Syndrome (MIC-CAP)		AR	*STAMBP*	STAM binding protein	Role in endosomal sorting and trafficking	Microcephaly (-2 to-8), severe ID, hypoplasia of distal phalanges, capillary malformations	*Stambp* -/- mice have brain lesions containing ubiuitinated protein aggregates.

Legend to tables 1-3

Known genetic causes of non-syndromic, syndromic microcephaly and additional abnormalities with MIM reference, cellular localisation and function, human phenotype and where available animal studies. In bold are genes and syndromes described in chapters in this thesis. AR: Autosomal Recessive, AD : Autosomal Dominant, ID: Intellectual Disability, IUGR: Intra-uterine Growth Retardation, FTT: Failure To Thrive, KO: knock out.

Chapter 2

Brain development and apoptosis

Microcephaly with simplified gyration, epilepsy and infantile diabetes linked to inappropriate apoptosis of neural progenitors

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Abstract

We describe a syndrome of primary microcephaly with simplified gyral pattern in combination with severe infantile epileptic encephalopathy and early onset permanent diabetes in two unrelated consanguineous families with at least 3 affected children. Linkage analysis revealed a region on chromosome 18 with a significant LOD score of 4.3. In this area, two homozygous non-conserved missense mutations in Immediate Early Response 3 Interacting Protein 1 (*IER3IP1),* were found in patients from both families. *IER3IP1* is highly expressed in fetal brain cortex and fetal pancreas and is thought to be involved in ER stress response. We reported one of these families previously in a paper on Wolcott-Rallison Syndrome (WRS). WRS is characterized by increased apoptotic cell death as part of an uncontrolled unfolded protein response (UPR). Increased apoptosis has been shown to be a cause of microcephaly in animal models. Autopsy specimen from one patient showed increased apoptosis in the cerebral cortex and pancreas beta-cells, implicating premature cell death as the pathogenetic mechanism. Both patient fibroblasts and control fibroblasts treated with siRNA specific for *IER3IP1*, showed an increased susceptibility to apoptotic cell death under stress conditions compared controls. This directly implicates *IER3IP1* in regulation of cell survival. Identification of *IER3IP1* mutations sheds light on the mechanisms of brain development and on the pathogenesis of infantile epilepsy and early onset permanent diabetes.

Introduction

Microcephaly is defined as small brain size detected by a head circumference more than 2.5 SD below the mean for age and sex. Microcephaly can be either primary i.e. congenital and present at birth, or progressive, i.e. caused by decrease of head circumference growth rate, which was normal until birth. The incidence of primary microcephaly ranges from 1/10.000 (consanguineous population) to 1/30.000 (non-consanguineous population)[1]. Primary microcephaly is considered to be caused by a brain developmental defect and at present the most widely accepted classification of primary microcephaly is Barkovich's et al. classification which is based on brain MRI imaging and genetic knowledge[2]. The classification includes microcephaly with a normal or a simplified cortex (MSG, MIM 603802**)**, with a thickened cortex (microlissencephaly, presumed to result form a neuronal migration disorder), or with polymicrogyria (presumed to be caused by a defect of cortical organization) and other structural malformations. MSG is presumed to derive from abnormal neuronal and glial proliferation or apoptosis[1]. Mutations in at least seven genes (*ASPM* (MIM, 605481)*, MCPH1* (MIM,607117), *CDK5RAP2* (MIM,608201)*, CEP152* (MIM, 613529), *CENPJ* (MIM 609279), *WRD62* (MIM,613583) *and STIL* (MIM,181590))[3] have been found that are causative for congenital primary microcephaly with normal or simplified gyration which is broadly indicated as "higher functioning", isolated microcephaly. These patients have near normal motor development with speech delay and moderately compromised cognition. Microcephaly with simplified gyral pattern only, in a recent review represented the largest group of patients with primary microcephaly and almost all of these had near normal function with only mildly delayed mile stones and none had epilepsy[5].

In contrast, primary microcephaly also occurs in syndromes with additional features. In such cases, patients have a poorer neurological function, with severe cognitive and motor impairment and epilepsy as common symptoms. For this group of patients mutations in at least 4 genes (*SLC25A19* (MIM 606521)[2], *ATR* (MIM 609215)[3], *ARFGEF2* (MIM 605371)[4], *RAB3GAP1* (MIM 602536)[4]) have been identified.

Darvish et al recently reviewed genetic causes of microcephaly in 112 Iranian families and came to the conclusion that the genetic cause is even more heterogeneous than previously thought[6].

A common function of many known microcephaly genes (both isolated and with additional features) include a role in microtubule and centrosomal

organisation, regulating mitosis, which is postulated to occur via a disruption in the balance of symmetrical and asymmetrical division of neural progenitors in the proliferative layer of the developing cortex i.e. the ventricular and sub-ventricular zone. This is dependent on correct centrosome and microtubule orientation to achieve the correct cleavage plane[7].

Despite this common theme, studies in animal models have shown that abnormal control of apoptosis can also be a pathogenic mechanism, therefore it has been proposed as a cause of human MSG but has thus far not been demonstrated in patients[2]. Apoptosis is an important regulatory mechanism of brain development specifically with the size of the forebrain[8]. Apoptosis is under complex control which results in activation of the caspase cascade and ultimately cell death.

The assumption that regulation of apoptosis is necessary for normal brain development has been demonstrated in $Mos^{+/-}$ mice haploinsufficient for Magoh, the exon junction complex component, which controls brain size by regulating neural stem cell division. Mutant mice show a microcephaly both due to an increase in neuronal apoptosis and a depletion of the intermediate neuron progenitor population[9]. Additionally, mice deficient in the RhoGTPase Rac1 produce a phenotype similar to primary microcephaly and also demonstrate an increase in apoptosis in the forebrain[10].

In contrast a reduction of apoptosis leads to increased brain size in caspase 9 knockout mice[11]. This further supports the hypothesis that a specific level of apoptosis is needed for normal brain development.

In some human disorders, deletion or duplications at the same locus can alternatively cause either micro- or macrocephaly demonstrating a dosage effect and suggesting a primary regulation of brain size, hence of neural proliferation at that same locus[12].

In some cases apoptosis is a response to stress conditions, for example if an excess of unfolded proteins accumulate in the ER (ER stress). In such cases a cascade of events, collectively called unfolded protein response (UPR) provide suppression of protein translation. If this UPR mechanism fails, apoptotic cascades are triggered leading to cell death[13].

Infantile onset permanent diabetes (PND, permanent neonatal diabetes, MIM 606176) has an incidence of approximately 1/260 000[14]. Unlike juvenile and adult onset diabetes it is not found to have an autoimmune cause. Instead the main mechanism is inappropriate apoptosis of Langerhans beta-islet cells, which are unable to cope with ER stress. This

has been described in Wolcott-Rallison Syndrome (WRS, MIM 226980), the most common cause of permanent neonatal diabetes in patients with consanguineous parents, which is caused by a mutation in the eukaryotic translation initiation factor 2-alpha kinase 3 gene, *EIF2AK3*[15]. Patients exhibit a combination of PND associated with multiple epiphyseal dysplasia and variable additional clinical features, including psychomotor retardation, hepatic dysfunction and MSG[16].

We have ascertained three patients from two consanguineous families with a striking overlapping phenotype. The affected children presented at an early age with the combination of MSG on MRI, infantile epileptic encephalopathy and infantile diabetes, resembling WRS and *EIF2AK3* mutations had been excluded. We have previously described one of these patients as representing a phenocopy of WRS, with unknown cause[16]. We then performed linkage analysis and identified overlapping areas of homozygosity by genomic SNP array analysis of the index patients. We hypothesized that these patients are suffering from a distinct genetic, presumably autosomal recessive syndrome and the results in this paper confirm this hypothesis.

Material and Methods

Patient material and RBI approval

All the family members and patient caretakers provided written informed consent for participation in the study and the genetic analysis was approved by the Erasmus Medical Centre research ethics committee.

Pathology study

A complete autopsy was performed in the male sibling of patient 2 (index patient 3, individual II:3 in figure 1B) shortly after his death at 26 months by one of the authors (Dr M. Jones) The material was fixed in 10% formalin, and samples were analyzed on 5-µm thick hematoxylin and eosin-stained sections of paraffin-embedded tissue. Immunohistochemistry for insulin was used in the pancreatic histological sections.

Brain was fixed in 10% buffered-formalin and sectioned. Samples from frontal, parietal, temporal and occipital lobes, deep nuclei as well as cerebellum, brain stem and spinal cord were submitted for histological evaluation. This was performed on 5-µm thick hematoxylin and eosin-stained sections of paraffin-embedded brain tissue. Myelin stain (Luxol-fast-blue) was also used.

SNP array, homozygosity mapping and linkage analysis

DNA from the index patients, the parents and unaffected siblings were hybridized to the arrays according to the Affymetrix standard protocol for the GeneChip Mapping 250K NspI array. Genotype data analysis was performed in Affymetrix GeneChip Genotyping analysis software (GTYPE) using the BRLMM algorithm. The obtained .CEL and .CHP files were used for homozygosity mapping in CNAG v3.0[17,18] and linkage analysis using Allegro in easyLINKAGE v2.1[19].

Sequencing analysis

Amplification reactions were performed in a total volume of 20 µl, containing 1x PCR buffer with Mg (Roche), 200 µM of each dNTP, 1 µM forward primer, 1 µM reverse primer, 0.1 units Fast Start Taq DNA polymerase (Roche) and 25 ng genomic DNA. PCR conditions: 5' 96^{0}C, 10 cycles of 30'' 96^{0}C, 30'' 68^{0}C (-1^{0}C/cycle), 60'' 72^{0}C, followed by 25 cycles of 30'' 96^{0}C, 30'' 58^{0}C, 60'' 72^{0}C and a final extension for 5' 72^{0}C.

PCR reactions were purified with ExoSAP-IT (USB). Direct sequencing of both strands was performed using Big Dye Terminator chemistry ver. 3.1 (Applied Biosystems). DNA fragment analysis was performed using capillary electrophoresis on an ABI 3130 Genetic Analyzer (Applied Biosystems) and the software package Seqscape (Applied Biosystems, version 2.1).

Microsatelite Markers Analysis

Amplification reactions were performed in a total volume of 20 µl, containing 1x PCR buffer with Mg (Roche), 200 µM of each dNTP, 1 µM forward primer, 1 µM reverse primer, 0.1 units Fast Start Taq DNA polymerase (Roche) and 25 ng genomic DNA. PCR conditions: 5' 96^{0}C, 35 cycles of 30'' 96^{0}C, 30'' 58^{0}C, 60'' 72^{0}C and a final extension for 5' 72^{0}C.

PCR products were diluted and pooled in panels and loaded on an ABI 3130 genetic analyser, with size standard LIZ 500 (Applied Biosystems) in formamide. Data was analyzed with Gene Mapper Version 2.1 software (Applied Biosystems).

Cell Culture and apoptosis

Fibroblast culture, RNA isolation cDNA synthesis

Fibroblasts were obtained from the certified Cell Repository of the department of Clinical Genetics, ErasmusMC, were routinely tested for mycoplasma and were cultured in 175 cm^2 culture flasks in Dulbecco's modified Eagle medium

(DMEM, Lonza Biowhittaker) until 80% confluence. Total RNA was extracted with Trizol reagent (GIBCO BRL Life Technologies,) and purified with Qiagen RNeasy mini columns (Qiagen,) according to manufacturers' protocols.

Reverse transcriptase was performed on 1 µg RNA in a total volume of 20 µl using iScript cDNA Synthesis Kit (BioRad) according to manufacturer's instructions.

Apoptosis Studies

Susceptibility to apoptosis studies were performed using patient cultured skin fibroblasts from the index patient of family 1, fibroblasts from a patient with confirmed Wolcott-Rallison syndrome bearing an *EIF2AK3* mutation[15] and 5 healthy control cells lines. Susceptibility to apoptosis of cultured skin fibroblasts was measured by fluorescent staining of active caspases using the Flica™ (Fluorescent-Labeled Inhibitor of Caspases) apoptosis multi-caspase detection kit (Immunochemistry Technologies, LLC), which makes use of an inhibitor sequence of active caspases, according to the manufacturer instructions. Fluorescent cells were scored before and after stress induction by 24 hours serum deprivation and then a further 24 hours exposure to 5 mM dithiothreitol (DTT) by two blinded investigators as percentage of apoptotic fibroblasts. The experiment was repeated three times and each time performed in triplicate. Necrosis vs apoptosis was tested by vital staining exclusion with propidium iodide.

Silencing experiments/RNA interference

Pre-designed siRNA pools targeting human *IER3IP1* (L-018948-01) (MIM 609382) and non-target control pools (D-001810-10-20) were purchased from Dharmacon and used to knock down *IER3IP1* in fibroblasts. siRNA was delivered to the fibroblasts via silentFECT Lipid Reagent (BioRad).

Silencing was confirmed at 24, 48 and 72 hours by RT-qPCR (primers: supplementary Table S2). Apoptosis was induced after 24 hours of serum deprivation, followed by addition of 1.25mM DTT for 24 hours; apoptosis was detected and percentages calculated using the Flica™ apoptosis detection kit as above.

Quantitative PCR (qPCR)

RT-qPCR was carried out using a KAPA SYBRH FAST qPCR Kit (Kapa Biosystems) in the CFX96 Real-Time system (BioRad). Thermal cycling conditions were as follows: denaturing step (95˚C for 3 minutes), followed

by 35 cycles of denaturing (95˚C for 5”), annealing and extension (60˚C for 30”). Fluorescence detection and data analysis were performed by BioRad CFX Manager 2.0. Experiments were performed in triplicate using as the reference UBE2D2 (Entrez GeneID 7322 [MIM 602962]) for gene expression normalization[20].

Induction by TNF-alpha

Fibroblasts were cultured as above to 80% confluency. In order to study induction of *IER3* (MIM 602996) and *IER3IP1* expression by TNF-alpha the cultures were exposed to two different conditions, 1st: standard culture medium (no induction with TNF alpha); 2nd: induction with 10ng/ml TNF alpha for 3 hours. After induction RNA was isolated with the Trizol LS protocol and RT-qPCR was performed for *IER3* and *IER3IP1* (primers in table S2).

Results

Patient description

Family 1

The index **patient 1** (Individual II:1 in Figure 1A) was a male infant born to consanguineous parents (fifth degree related, second cousins) of Moroccan origin (Figure 1A top panel). They also have two healthy daughters and a healthy son. The patient was born after a normal pregnancy and at birth his head circumference measured 2.5SD below the mean. At 2 months of age he presented with microcephaly greater than 3SD below the mean, developmental delay, hypotonia, epilepsy, obesity, presumed hypogonadism and infantile diabetes. At 2 months of age the seizures were a combination of focal seizures with secondary generalisation and generalised seizures. Electroencephalographs (EEG) showed high voltage asymmetric multifocal activity with abnormal background progressing into burst suppression on sleep EEG at 4 months and hypsarrythmia at 7 months. The MRI showed a simplified gyral pattern of the cerebral cortex and delayed myelination, no overt cerebellar abnormality (Figure 2A-C). Additional metabolic and infection screening, including a muscle biopsy, was negative. *ARX* sequencing was normal. The diabetes and epilepsy were difficult to control despite treatment with clonazepam, vigabatrin and sodium valproate. Patient 1 died at the age of 18 months of a lower respiratory tract infection complicated by therapy-resistant epilepsy and diabetes. A post-mortem exam was declined by the family. His clinical history has been reported by de Wit et al[16].

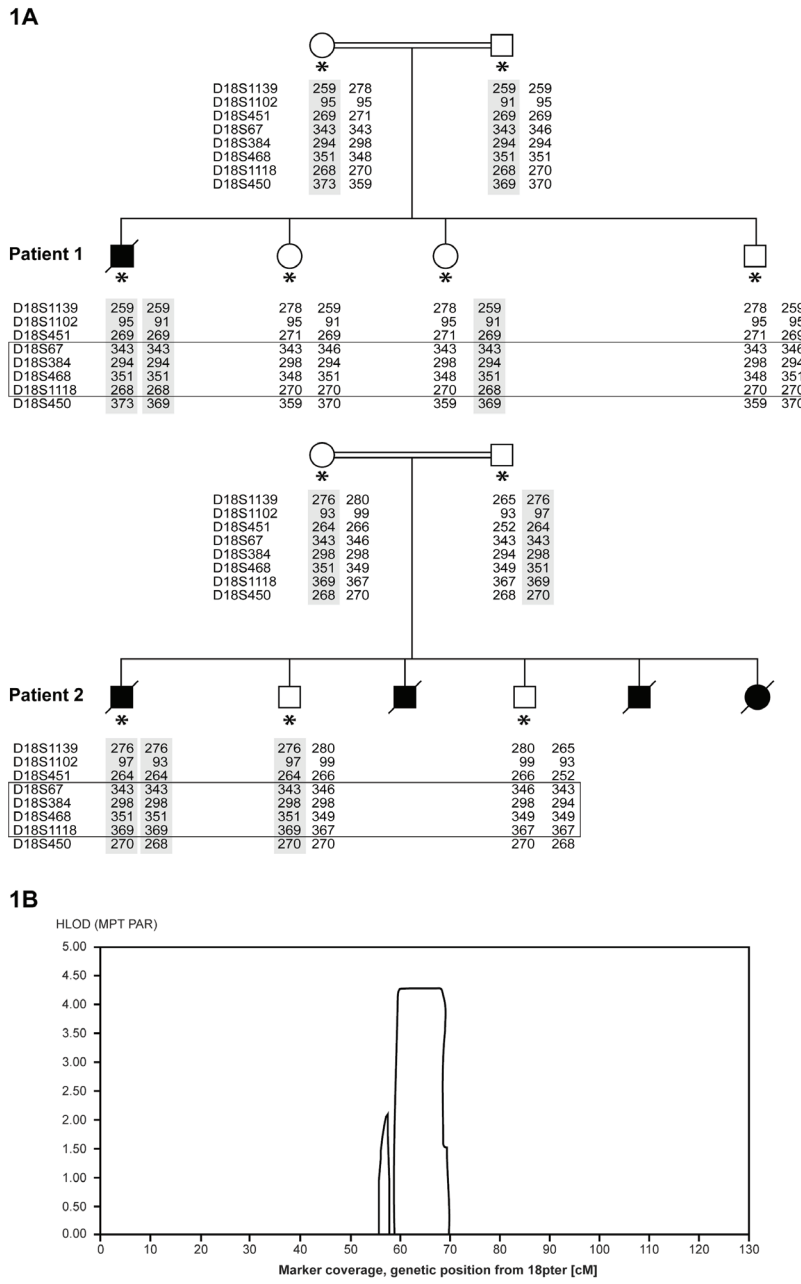

Figure 1. Family pedigrees and linkage data (A,B) Simplified genealogical trees of two unrelated families with MSG, epilepsy and PND. Filled symbols represent affected patients. A double line represents consanguinity. An asterix represents subjects analysed by SNP arrays and included in the linkage analysis. The results of microsatellite markers analysis confirming homozygosity are also shown. The shaded areas are the alleles containing the mutation and the box represents the area.

Family 2

Index **patient 2** (Individual II:1 in Figure 1B) was the fourth affected child (there were also 2 similarly affected sons and 1 affected daughter) from a presumed consanguineous family in Argentina (Figure 1A, lower panel). He was born at term gestation after a normal pregnancy. Infantile diabetes and microcephaly, with a brain size greater then 3SD below the mean, were noted at birth. MRI performed at 2 months showed microcephaly with a simplified gyral pattern (Figure 2). The seizures were generalised tonic-clonic and myoclonic in type. One EEG was performed at 2 years of age, which showed hypsarrythmia. After multiple hospital admissions with seizures and poor diabetic control, patient 2 died at 27 months from a lower respiratory tract infection complicated by epilepsy and diabetes. In the family three more siblings died of a similar disorder (reported as West syndrome and infantile diabetes) but medical records are not available.

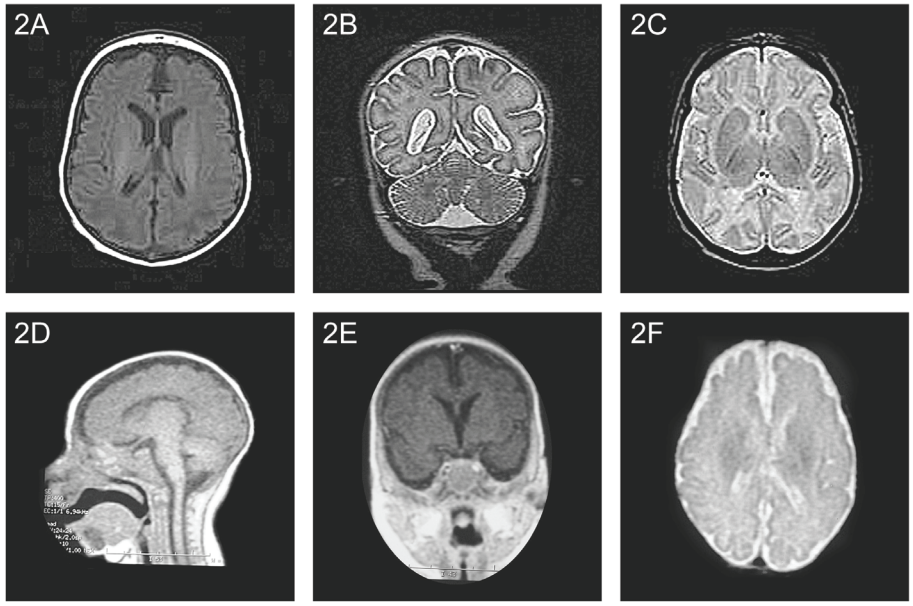

Figure 2. Patient brain MRIs (A-C), MRIs from patient 1 performed at 1 year of age. (2A) T1-weighted image showing a simplified gyral pattern and increased intra-cranial space with near-normal ventricular size. (2B-C) T2 weighted coronal and axial view showing equal involvement of parietal lobes, normal cerebellum and basal ganglia with delayed myelination. (2D-F) Low-resolution MRIs from patient 2 at approximately 7 months of age. (2D) Sagittal T1 weighted view showing a thin but present corpus callosum and normal brain stem, (2E) T1-weighted coronal view through the hippocampus showing simplified gyration and hippocampal hypoplasia and (2F) T2- weighted view, demonstrating the simplified gyral pattern and normal ventricular size.

Pathology findings

Post mortem macroscopic examination (performed after his death at 26 months) of one similarly affected brother of patient 2 from the Argentinian family (index **patient 3**, Individual II:3 in figure 1B), confirmed extreme microcephaly (weight 175g) compared to an age matched control (970g) and simplified gyral pattern throughout the cortex with the temporal tips and interhemisperic parietal lobes showing extreme simplification (Figure 3A-D). The cerebellum while relatively large compared to the cortex was also small (weight 59g, age

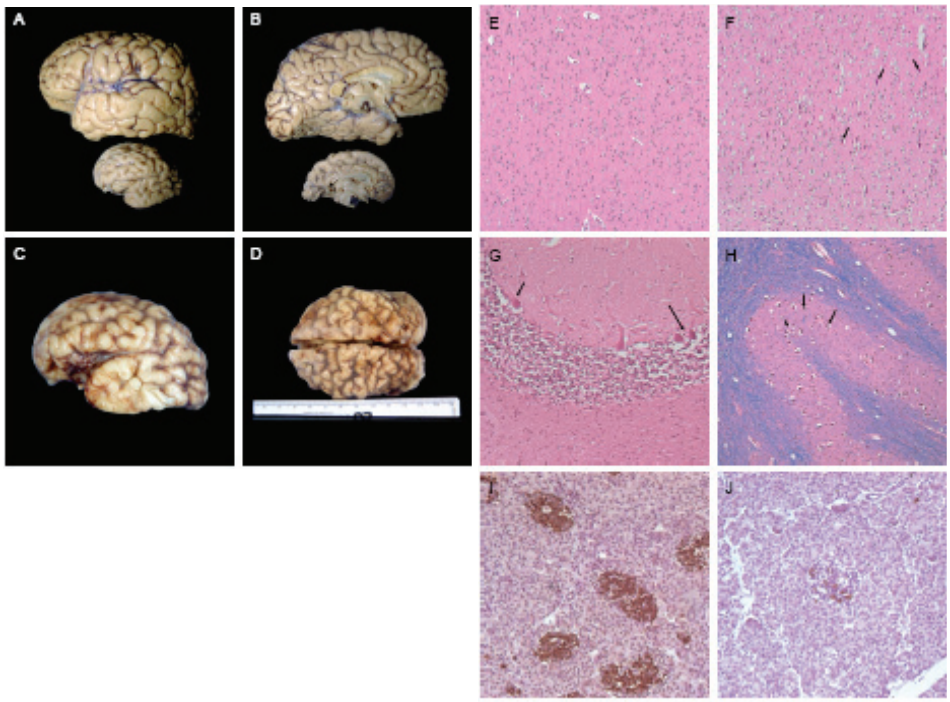

Figure 3. Brain macroscopy and histopathology. (3A-B) Macroscopic view in real proportions of normal age-matched brain (upper specimen) compared with index **patient 3** (individual II:3 in Figure 1B) post-mortem brain (lower specimen); note the extreme microcephaly and simplified gyration looking almost lissencephalic in its medial surface. (3C-D) macroscopic photographs of **patient** 3 brain, showing shallow and insufficient in number sulci over the whole brain surface, but more apparent in the temporal and parietal lobes. Note photos 3C and D are not in scale with 3A and B. (3E-J) Histopathology. (3E), Histopathology of normal cerebral frontal cortex (Age- matched to **patient 3**), (3F) **patient 3** histopathology of the cerebral cortex showing numerous apoptotic neurons (arrows) an some apoptotic glial cells (H&E x 20). (3G) Cerebellar folia: Purkinje neurons are apoptotic (arrows) and internal granule cell layer is diminished (H&E x 20) (3H) Nearly all neurons and the inferior olivary nuclei are apoptotic. (3J) **Patient 3** pancreas stained for insulin showing depletion of insulin producing islet cells in comparison to a normal pancreas (3I).

matched control 125g). Microscopy revealed reduced number of neurons in the brain cortex, mainly of layers 1 and 4, hypomyelination and remarkable apoptosis. Many picnotic dead cells were also observed in the inferior olivary nucleus and in the cerebellum while the pancreas showed few and small islets with few insulin positive beta cells (Figure 3E-G).

Molecular analysis

The 250K SNP array data from the index patients (probands), unaffected sibs and parents were analysed for loss of heterozygosity (LOH) regions using CNAG and the pedigrees were subsequently used for easyLINKAGE[19]. In both families multiple areas of apparent LOH larger than 1 Mb were observed in the probands, suggesting homozygosity and compatible with the reported consanguinity. We observed one LOH region on chromosome 18 overlapping between patients from the two families. We then performed a linkage analysis on the SNP array data and found a significant LOD score of 4.3 linked to the same region on chromosome 18q, spanning a 10Mb region and containing 43 genes (NCBI built 36.3). The region was confirmed as homozygous in the affected and heterozygous in the unaffected siblings by microsatellite markers (Figure 1A and supplementary Figure S1).

The genes in the linkage area were prioritised for sequencing using available expression data from GEO profile. In total 12 genes were found to be expressed both in the brain and pancreas and these were all sequenced (Table S1). In both affected individuals from the two pedigrees a homozygous variation in the Immediate Early Response-3 Interacting Protein-1 (*IER3IP1*) was found In index patient 1 (Individual II:1 in Figure 1A) a homozygous missense change was found in exon 1, c.62T>G, (NM_016097.3) causing a change in amino acid at position 21 from Valine to Glycine (p.Val21Gly). In index patient 2 (Individual II:1 in figure 1B) a homozygous missense change was found in exon 3, c.233T>C. This causes a change in amino acid at position 78 from Leucine to Proline (p.Leu78Pro) (Figure 4A). The parents in both families were confirmed heterozygotes for the changes, while the non-affected sibs were confirmed either heterozygotes or had no changes. Unfortunately, no DNA of index patient 3 was available. Neither of these changes was annotated in dbSNP 132 or the 1000 genome database, or was found in 300 ethnically matched control chromosomes, indicating in each case an allele frequency lower than 0.3%.

No change predicted to be pathogenic was observed in any of the other genes in the linkage area in the affected subjects.

In silico IER3IP1 function prediction and mutation effect

IER3IP1 encodes a small polypeptide protein of about 10 kDa[21]. Protein localisation studies of IER3IP1 and the yeast homologue Yos1p localise the protein to the ER and Golgi and suggest its involvement in control of COP-II vesicle budding[21,22]. The protein sequence predicts one N-terminal hydrophobic domain possibly including a signal peptide sequence[23], a hydrophilic glycine-rich domain containing (G-patch), putatively interacting with RNA, and a C-terminal second hydrophobic trans-membrane domain (Figure 4A). Signal P 3.0 server[23] predicts a hydrophobic domain, including a signal peptide which is required for targeting the peptide to the endoplasmic reticulum (ER). The p.Val21Gly change is located in this first domain and introduces a new glycine just before the G-patch. Signal P 3.0 suggests that this mutation is very close to a putative peptide cleavage site, possibly interfering with the cleavage of the signal sequence[23]. Preliminary data show that IER3IP1 is undetectable in fibroblasts from patient 1 (supplementary Figure S5). The p.Leu78Pro change is located in the second trans-membrane domain. Using Project HOPE and tools for predictions of amino acid changes on protein structure, both mutations are predicted to cause a change in protein structure and are predicted to be within a hydrophobic/trans-membrane domain[24,25]. The p.Leu78Pro is predicted to be within an alpha helix and is predicted to have severe effects on the structure of the protein (supplementary Figure S4).[24,25] *In silico* analysis of the p.Val21Gly by the Grantham Matrix Score[26], Polyphen-2[27], SIFT[28], SNAP[29] and Pmut[30] programmes predicted in a pathogenic/non-neutral effect, (see supplementary Table S3). The p.Leu78Pro was predicted to be pathogenic/non-neutral by the Grantham Matrix Score[26], Polyphen-2[27], SNAP[29] and Pmut[30] programmes (see supplementary Table S3). Both mutations are in a highly conserved area among vertebrates (Figure 4A). Although very little has been published about *IER3IP1*, search in several *in silico* expression databases (GEO profile, GenePaint, Allen Brain Altlas, MGI, EMBL-EBI) brought additional information about its putative function. The mouse homolog *Ier3ip1* shows a high level of expression during early gestation (E9.5 and E11.5, compared to E13.5) which corresponds to the period of proliferation of neural progenitors. In the developing mouse at E14.5 there is high expression of *Ier3ip1* in the developing mouse cortex in the ventricular/sub-ventricular zone at the site of neurogenesis[31] (Figure 4B).

Expression in embryonic pancreas peaks at E12.5 and lowers at E13.5 and E14.5 (GEOprofile, GenePaint). There also appears to be expression in the lungs and the kidneys. The *IER3IP1* mRNA is putatively translated into the endoplasmic reticulum, suggesting that it functions indeed as a membrane bound protein (EMBL-EBI database). In adult mouse brain its

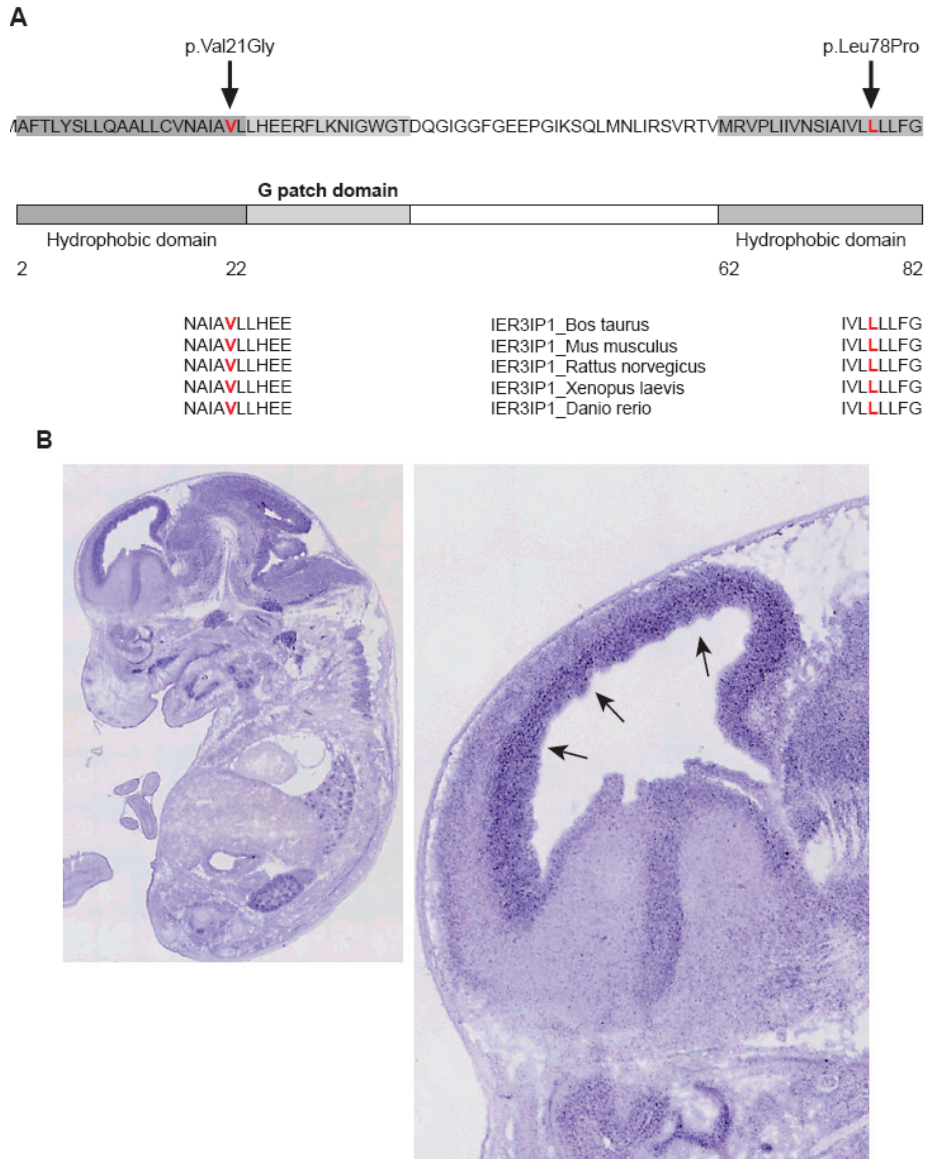

Figure 4. *IER3IP1* sequence and expression in developing brain. (4A) Schematic representation of the human IER3IP1 protein showing the sequence, the predicted protein domains and the location of the p.Val21Gly and the p.Leu78Pro mutations. Cross species conservation of IER3IP1 in the areas of the mutations is shown at the bottom of the diagram. Both the predicted amino acid changes are in a highly conserved area of the gene and in the hydrophobic/transmembrane domains and are depicted in red. (4B) Expression of *Ier3ip1* at E14.5 days in the whole mouse embryo (left); zoomed in, in the right panel is the mouse brain, with arrows pointing to increased expression in the ventricular and sub-ventricular zone at the site of neurogenesis[24].

expression is low and essentially limited to the cerebellum, hippocampal area, midbrain and thalamus, with low, albeit detectable expression in the cortex (Allen Brain atlas). There is also additional evidence that *IER3IP1* is down-regulated by miR-34a[32]. Once trans-activated by p53, this micro-RNA is known to promote apoptosis[32]. This data suggest a putative function for IER3IP1 in protecting neural progenitors in the proliferative zone of the embryonic forebrain from apoptosis.

Apoptosis tests in vitro

As a next step we tested the general tendency of cultured fibroblasts to undergo apoptosis by use of a test for active caspases after induction of stress by dithiothreitol[33].

Fibroblasts in an individual with an IER3IP1 mutation showed an significantly increased tendency to undergo apoptosis after treatment with DTT in comparison to control cells ($p \leq 0.001$, unpaired t-test, SPSS version 17.0) (Figure 5A). The rate of apoptosis was similar to the cells of a patient with confirmed Wolcott-Rallison syndrome[16]. However, considering the p.Val21Gly missense mutation could synthesize an abnormal protein, increased apoptosis could be the result of a putative ER stress induced by a less functional IER3IP1 protein accumulating in the ER and not be linked to a specific function of IER3IP1 in protecting the cells from apoptosis. We therefore tested the susceptibility to stress induced by DTT after silencing of *IER3IP1* in control fibroblasts by specific small interfering RNA. RT-q-PCR of the *IER3IP1* transcript showed efficient down-regulation, i.e. diminished predicted IER3IP1 synthesis (Figure 5B, left panel) In cells where *IER3IP1* had been knocked-down a significant increase ($p \leq 0.001$, unpaired t-test, SPSS version 17.0) in apoptosis was observed after stress induced by 1.25mM DTT, when compared to the same cell line treated with control siRNA (Figure 5B, right panel). The DTT concentration in these experiments was lower because the siRNA treatment made cells more susceptible to apoptosis (also seen in control experiments). No difference in the level of apoptosis was observed in the absence of stress or when the cells were only exposed to serum deprivation (data not shown).

These results demonstrate that IER3IP1 is needed *in vitro* to protect cells from the consequences of stress induced by denaturing agents like DTT.

TNF-alpha experiments

In the gene annotation databases, the IER3IP1 protein is considered as a putative interactor of IER3, the Early Immediate Response-3 regulating acute stress response and cell death through the TNF-alpha pathway[34],

but any evidence for the human protein is lacking. In control fibroblasts, we found that expression of *IER3* mRNA was approximately 10 fold higher after induction with TNF-alpha in comparison to standard culture conditions (p<0.0001, unpaired t-test, SPSS version 17.0) (figure 5C). To test if *IER3*

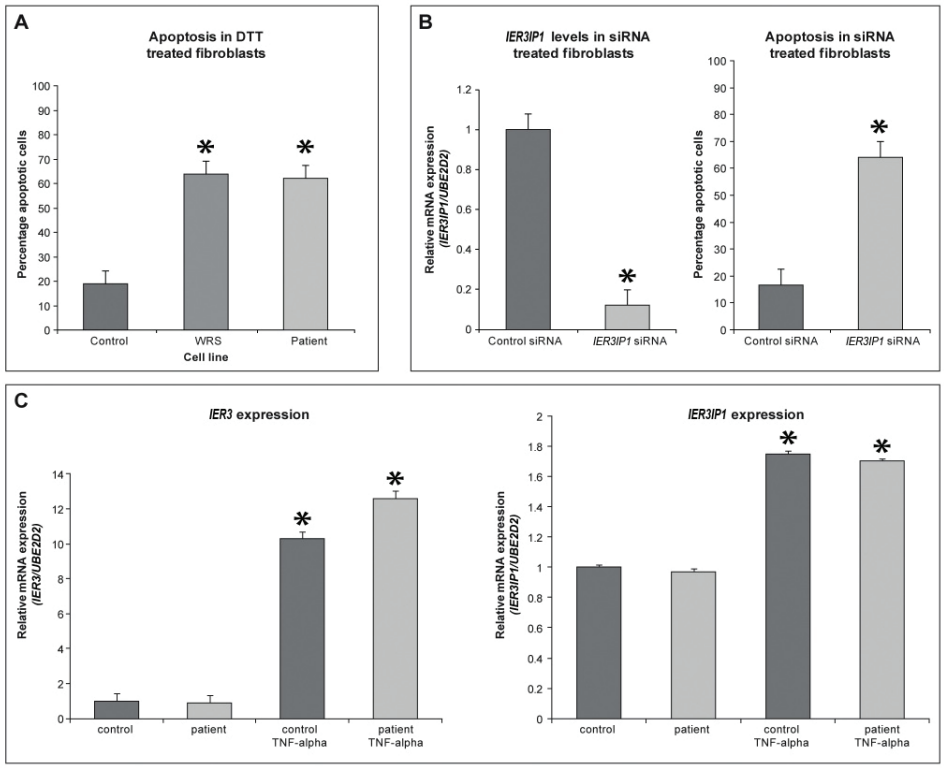

Figure 5. Susceptibility of cultured fibroblasts to apoptosis and TNF-alpha stimulation. (5A): Patient cells are more susceptible to apoptosis similar to cells from a known WRS patient, when treated with 5mM DTT in comparison to 5 control cell lines (p≤0.001, unpaired t-test, SPSS version 17.0). Values represent a mean of 3 experiments +/-SEM. (5B**)**: *IER3IP1* expression levels in control fibroblasts are approximately 10 fold decreased after addition of *IER3IP1* siRNA in comparison to control siRNA (p≤0.001, unpaired t-test, SPSS version 17.0) (left panel). Control fibroblasts treated with *IER3IP1* siRNA also demonstrate a significantly increased susceptibility to apoptosis (p≤0.001, unpaired t-test, SPSS version 17.0) when treated with 1.25mM DTT in comparison to control fibroblasts (right panel). Values represent a mean of 3 experiments +/-SEM. (5C) *IER3* mRNA levels are approximately 10 fold increased in patient and control cells after addition of TNF-alpha (left panel) (unpaired t-test SPSS version 17.0,P<0.0001). *IER3IP1* levels are approximately 1.5 fold increased in patient and control fibroblast cell lines after addition of TNF-alpha (p<0.001, unpaired t-test SPSS version 17.0)(right panel). Stars represent a significant p<0.01 difference.

and *IER3IP1* are similarly regulated we looked at the expression of *IER3IP1* after the addition of TNF-alpha. There was an approximate 1.5 fold increase in expression of *IER3IP1* mRNA after induction with TNF-alpha both in patient and control fibroblasts ($p<0.001$, unpaired t-test, SPSS version 17.0) suggesting that *IER3IP1* is under similar control as *IER3* (Figure 5C).

Discussion

We have described a syndrome consisting of MSG, PND, severe apoptotic brain and infantile epileptic encephalopathy in patients from two separate families, associated with mutations in the *IER3IP1*.

Collectively, the linkage and *IER3IP1* sequence data together with the expression pattern during brain development and its involvement in regulation of apoptosis *in vitro* indicate that the mutations observed in our patients are causally related to their disease.

The congenital microcephaly and insulin dependent diabetes can be explained by the presence of abnormal levels of neuronal apoptosis in brain and pancreas specimen from the sibling of patient 2. In this brain there is additional evidence for insufficient myelination, which suggests either that early common progenitors of both neural and glia lineage are depleted or that maturation of the oligodendrocyte progenitors is blocked. Consistent with the hypothesis of progenitor depletion and block in maturation, the pattern of *Ier3ip1* in mouse brain at embryonic day 14.5 shows selective expression at the proliferative ventricular and sub-ventricular zone, where the common neuro-glial progenitors are located before differentiation in to separate lineages. Although naturally occurring cell death represents a final pathogenic mechanism of many disorders, apoptosis is an essential and impressive feature of cortical brain development. During neurogenesis, about 70% of progenitor cells in the ventricular and sub-ventricular zone undergo apoptosis, this number being similar to that of post-mitotic cells, leading to the suggestion that apoptosis controls stem cell population[35]. Abnormal increase of apoptosis in the *IER3IP1* patients could either be the result of insufficient regulation of mitosis and differentiation of neural progenitors or insufficient protection from pro-apoptotic stimuli. Distinction between these two mechanisms might be particularly important for the identification of pro-apoptotic stimuli extrinsic to the cell metabolism, like oxidative stress, which might be preventable[36]. Fibroblasts from an individual with an *IER3IP1* mutation show increased rates of apoptotic cell death when exposed to DTT. Additionally, silencing of the *IER3IP1* leads to

increase in apoptosis rate in control fibroblasts after DTT treatment. On the one hand these results link the effect of the mutation with the onset of apoptosis, on the other hand they suggest that IER3IP1 intervenes under "stress" stimuli. IER3IP1 is putatively involved in cell differentiation and cell death processes, under regulation of transcription factors TNF-alpha and Sp1[34]. If IER3IP1 normally protects the cell from stimuli inducing apoptosis, the modest albeit significant response to TNF-alpha places human IER3IP1 in the "death receptors" cascade[37]. IER3IP1 has also a reported role in ceramide apoptosis in neurons[38].

IER3IP1 is a highly conserved protein that is found in humans, mice, zebrafish (Figure 4A) and yeast (homolog to Yos1p) and is highly expressed in the developing cortex and in the beta cells of the pancreas. IER3IP1 putatively interacts with IER3 and, based on its sequence homology to the yeast protein Yos1p and preliminary experimental evidence, it is an endoplasmic reticulum protein containing at least one trans-membrane domain and mediating vesicular transport between Golgi and ER[33]. However, it also contains a G-patched domain present in RNA-associated proteins.

IER3IP1 has a similarity in name to and may interact directly with IER3, although there is no available experimental evidence published to prove this. IER3 is a highly conserved protein consisting of 156 amino acids in humans and consists of a putative trans-membrane domain and a nuclear localisation site. IER3 is described as having both a pro and anti-apoptotic role[39,40].

An elevated expression of IER3 in hepatocytes and pancreatic tumour cell lines triggers apoptosis in response to various apoptotic stimuli. IER3 has also been reported to be part of the DNA damage-induced and p53mediated apoptosis in rodent and human cells[40].

Our experiments showing increased expression of IER3 and IER3IP1 after induction with TNF-alpha could provide clues that IER3IP1 regulates apoptosis via interaction with IER3, either through cooperation or through interference with IER3 function.

We therefore conclude that *IER3IP1* is an important gene,, involved in the development of microcephaly and diabetes via dysregulation of apoptosis.

The role of *IER3IP1* in cortical development and pathogenesis of epilepsy and diabetes is obscure but it seems essential from the

early stages of development, such as the period of neural progenitor proliferation. It is unknown when the diabetes starts in the patients because there has been no way to test it prenatally, but the presence of microcephaly with simplified gyration indicates a crucial role already during gestation. Severe infantile epileptic encephalopathy is very unusual in primary microcephaly and has only been reported in patients with *WDR62* mutations[41]. In these patients, however, the structure of the cortex is deeply abnormal and shows both migration and organization defects, both high risk factors for epilepsy. The rare combination of primary microcephaly with severe infantile epilepsy has been observed in *PNKP* mutations[42]. In this disorder, seizures and microcephaly are not related to degenerative changes, but to probable defects in DNA repair mechanisms of nucleotide excision or single/double stranded breaks. Interestingly, mouse neuronal cells show increased apoptosis rates when *PNKP* is silenced by siRNA.

Apoptosis or programmed cell death is also end point of the unfolded protein response. WRS, which shows phenotypic similarity to our patients' syndrome, is caused by a mutation in the *EIF2AK3*, which encodes a kinase in the ER membrane, one of four that phosphorylates EIF2a (also known as Perk in mice), having an important role in the unfolded protein response[15]. When a protein is misfolded, EIF2a is phosphorylated resulting in lower levels of translation initiation of the protein and eventually prevention from apoptosis. This is proven by the Perk knock out mice, which have an increased rate of apoptosis and destruction of beta cells causing a similar phenotype to WRS in humans[43]. It is interesting that both of the *IER3IP1* mutations found are in the putative hydrophobic regions which therefore seem to be functionally very relevant. It is possible that the role of IER3IP1 in protecting from apoptosis is also played by regulating and/or preventing nascent protein (mis)folding in the ER membrane, at the lumen and thus preventing activation of the UPR response. This and other questions might be addressed by future protein studies on subcellular localization and on identification of interacting partners. These studies will also be instrumental in the understanding of severe infantile epilepsy and infantile diabetes.

Supplementary data (5 figures and 3 tables)

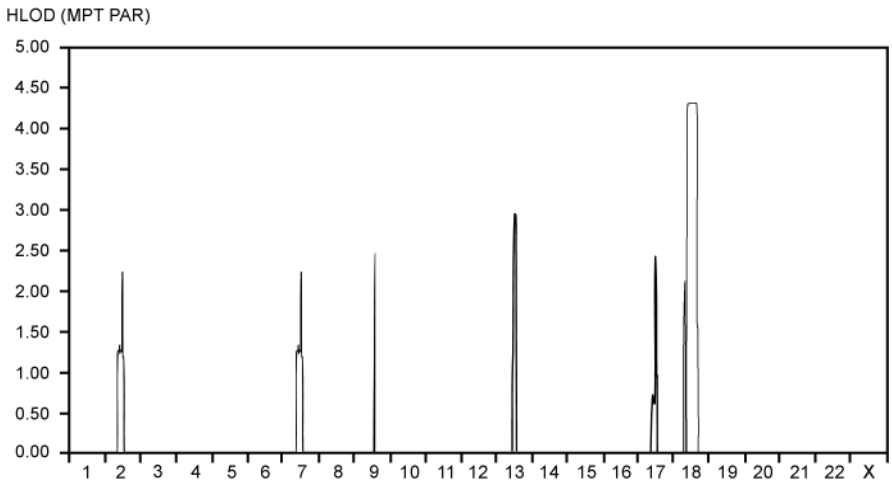

Figure S1. Results of the whole genome scan from EasyLINKAGE.

Significant peaks (>3) were found on chromosomes 13 and 18 (X axes). The highest was on chromosome 18 (4.3). However when these were analysed individually only the peak on chromosome 18 remained significant.

Figure S2. HOPE project prediction of IER3IP1 mutation (p.Val21Gly) effects on the protein.

We observed a mutation of a Valine into a Glycine at position 21. The figure below shows the schematic structures of the original (left) and the mutant (right) amino acid. The backbone, which is the same for each amino acid, is coloured red. The side chain, unique for each amino acid, is coloured black.

The residue is located in a region annotated in the Uniprot database as a transmembrane domain. The new mutant residue is smaller than the wild-type residue. This can disturb either the contacts with the other

transmembrane domains or with the lipid-membrane. The wild-type residue was more hydrophobic than the new mutant residue. This can affect the hydrophobic interactions within the core of the protein or with the membrane lipids. The mutation introduces a glycine at this position. Glycines are very flexible and can disturb the required rigidity of the protein on this position.

The wild-type and new mutant amino acids differ in size. The new mutant residue is smaller than the wild-type residue. The mutation will cause an empty space in the core of the protein (or protein-complex). The hydrophobicity of the wild-type and new mutant residue differs. The mutation will cause loss of hydrophobic interactions in the core of the protein (or protein-complex).

Figure S3. HOPE project prediction of IER3IP1 mutation (p.Leu78Pro) effects on the protein.

We found a mutation of a Leucine into a Proline at position 78. The figure below shows the schematic structures of the original (left) and the mutant (right) amino acid. The backbone, which is the same for each amino acid, is coloured red. The side chain, unique for each amino acid, is coloured black

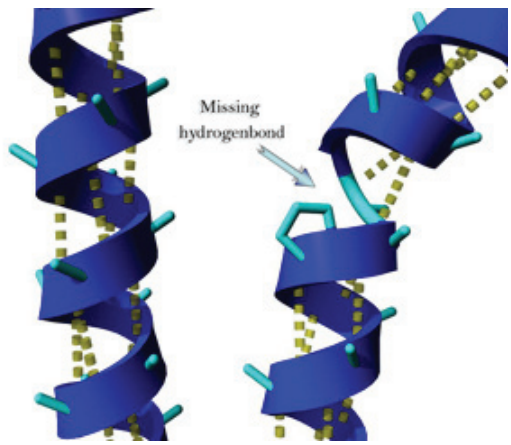

Figure S4. Disruption of alpha-helix in p.Leu78Pro.

The residue is located in a region annotated in the Uniprot database as a transmembrane domain. The new mutant residue is smaller than the wild-type residue. This can disturb either the contacts with the other transmembrane domains or with the lipid-membrane. The wild-type residue is predicted (by the PHDacc DAS-server) to be located in an α-helix. Proline disrupts an α-helix when not located at one of the first two positions. In case of the mutation at hand, the helix will be disturbed and this can have severe effects on the structure of the protein.

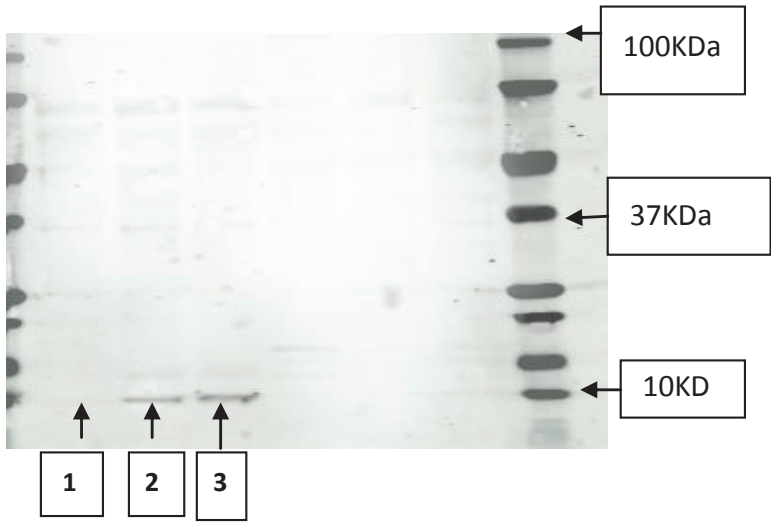

Figure S5. Western blot of IER3IP1.

Western blot showing IER3IP1 is detectable in 2 control fibroblast homogenates (lane 2 and 3) using specific custom made polyclonal (rabbit anti human) antibodies raised against the whole polypeptide (SciLight Biotechnology Ltd.), but not in fibroblasts from index patient 1, (individual II:1 in Figure 1A), (lane 1).

On the SDS-polyacrylamide gel, the molecular weight marker of 10KDa indicates the level of the IER3IP1 protein band. Protein homogenate in each lane was obtained from about 7 x 10^6 cultured cells, equivalent of one T175 cm^2 flasks. The results were reproduced in two separate experiments. The last 3 lanes in the picture are empty.

Table S1. List of sequenced genes in shared homozygous area.

Ensemble Gene id	Description
ENSG00000152240	**HAUS** augmin like complex subunit 1 [Source:HGNC Symbol;Acc:25174]
ENSG00000197046	**SIGLEC15** sialic acid binding Ig-like lectin 15 [Source:HGNC Symbol;Acc:27596]
ENSG00000152234	**ATP5A1** ATP synthase, H+ transporting, mitochondrial F1 complex, alpha subunit 1, cardiac muscle [Source:HGNC Symbol;Acc:823]
ENSG00000167220	**HDHD2** haloacid dehalogenase-like hydrolase domain containing 2 [Source:HGNC Symbol;Acc:25364]
ENSG00000132872	**SYT4** synaptotagmin IV [Source:HGNC Symbol;Acc:11512]
ENSG00000134049	**IER3IP1** immediate early response 3 interacting protein 1 [Source:HGNC Symbol;Acc:18550]
ENSG00000134049	**SLC14A1** solute carrier family 14 (urea transporter), member 1 (Kidd blood group) [Source:HGNC Symbol;Acc:10918]
ENSG0000015229	**PSTPIP2** proline-serine-threonine phosphatase interacting protein 2 [Source:HGNC Symbol;Acc:9581]
ENSG00000175387	**SMAD2** SMAD family member 2 [Source:HGNC Symbol;Acc:6768]
ENSG00000152217	**SETBP1** SET binding protein 1 [Source:HGNC Symbol;Acc:15573]
ENSG00000152242	**C18orf25** chromosome 18 open reading frame 25 [Source:HGNC Symbol;Acc:28172]

Table S2. Primers used for RT-q-PCR experiments (Figure 5).

Primer pair	Forward	Reverse
IER3IP1 spanning exon 2-3	CAGGGAATTGGTGGATTTGG	TGGCACTCTCATCACGGTTCT
IER3 spanning exon 1-2	TCTTCACCTTCGACCCTCTC	GGGTTCGGTTCCTCGACT
UBE2D2 spanning exons 5 and 6	GATCACAGTGGTCTCCAGCA	CGAGCAATCTCAGGCACTAA

Table S3. Summary of mutation prediction program output for the p.Val21Gly and p.Leu78Pro mutations in IER3IP1.

	p.val21Gly	**p.Leu780Pro**
Polyphen2 HumVar	Probably damaging (0.978)	Probably damaging (0.956)
Polyphen2 HumDiv	Possibly damaging (0.830)	Possibly damaging (0.634)
Grantham Matrix Score	109 (pathological)	78 (pathological)
SIFT	Affect protein function (score 0.0)	Tolerated (score 0.16)
SNAP	Non-Neutral	Non-Neutral
pmut	Pathological (0.99)	Pathological (0.8668)
Mutation taster	Disease causing (0.99)	Not available

References

1. Woods C.G, Bond J, and Enard W (2005) Autosomal recessive primary microcephaly (MCPH): a review of clinical, molecular, and evolutionary findings. Am. J. Hum. Genet.76, 717-28.

2. Barkovich A.J, Kuzniecky R.I, Jackson G.D, Guerrini R, and Dobyns W.B (2005) A developmental and genetic classification for malformations of cortical development. Neurology 65, 1873-1887.

3. Kaindl A.M, Passemard S, Kumar P, Kraemer N, Issa L, Zwirner A, Gerard B, Verloes A, Mani S, and Gressens P (2010) Many roads lead to primary autosomal recessive microcephaly. Progress in Neurobiology 90, 363-383.

4. Guerrini R, Dobyns W.B, and Barkovich A.J (2009) Abnormal development of the human cerebral cortex: genetics, functional consequences and treatment options. Trends in Neuroscience 3, 154-162.

5. Basel-Vanagaite, L., and Dobyns, W.B (2010) Clinical and brain imaging heterogeneity of severe microcephaly. Pediatr. Neurol. 43, 7-16.

6. Darvish H, Esmaeeli-Nieh S, Monajemi G.B, Mohseni M, Ghasemi-Firouzabadi S, Abedini S.S, Bahman I, Jamali P, Azimi S, Mojahedi F, et al (2010) A clinical and molecular genetic study of 112 Iranian families with primary microcephaly J. Med. Genet. 47,823-828.

7. Nigg E, and Raff J.W (2009) Centrioles, centrosomes, and cilia in health and disease. Cell 139, 663-78.

8. Haydar T.F, Kuan C.Y, Flavell R.A, and Rakic P (1999). The role of cell death in regulating the size and shape of the mammalian forebrain. Cereb. Cortex. 9, 621-6.

9. Silver D.L, Watkins-Chow D.E, Schrech K.C, Pierfelice T.J, Larson D.M, Burnetti A, Liaw H.J, Myung K, Walsh C.A, Gaiano N, et al (2010) The exon junction complex component Magoh controls brain size by regulating neural stem cell division. Nat. Neurosci. 13, 551-558.

10. Sanno H, Shen X, Kuru N, Bormuth I, Bobsin K, Gardner H.A, Komljenovic D, Tarabykin V, Erzurmulu R.S, and Tucker K.L (2006). Control of postnatal apoptosis in the neocortex by RhoA- subfamily GTPases determines neuronal density. J. Neurosci. 30, 4221-4231.

11. Kuida K, Haydar T.F, Kuan C.Y, Taya C, Karasuyama H, S.-S.Su, Rakic M, and Flavell, R.A. (1998) Reduced Apoptosis and Cytochrome c-Mediated Caspase Activation in Mice lacking Caspase 9. Cell, 94, 325-337.

12. Brunetti-Pierr N, Berg J.S, Scaglia F, Belmont J, Bacino C.A, Sahoo T, Lalani S.R, Graham B, Lee B, Shinawi M et al (2008) Recurrent reciprocal 1q21.1 deletions and duplications associated with microcephaly or macrocephaly and developmental and behavioral abnormalities. Nat. Genet. 40, 1466-71.

13. Merksamer P.I and Papa R.R (2010) The UPR and cell fate at a glance. J. Cell. Sci. 123, 1003-1006.

14. Slingerland A.S, Shields B.M, Flanagan S.E, Bruining G.E, Noordham K, Gach A, Miynarski W, Malecki M.T, Hattersley A.T and Ellard S (2009) Referral rates for diagnostic testing support an incidence of permanent neonatal diabetes in three European countries of at least 1 in 260,000 live births. Diabetologia 52, 1683-5.

15. Rubio-Cabezas O.I, Patch A.M, Minton J.A.L, Flanagan S.E, Edghill E.L, Hussain K, Balafrej A, Deeb A, Buchanan C.R, Jefferson I.G et al (2009) Wolcott-Rallison syndrome is the most common genetic cause of permanent neonatal diabetes in consanguineous families. J. Clin. Endocrinol. Metab. 94, 4162-70.

16 de Wit M.C.Y, de Coo I.F.M, Julier C, Delephine M, Lequin M.H, van de Laar M, Sibbles B.J, Bruining G.J, Mancini G.M.S (2006) Microcephaly and simplified gyral pattern of the brain associated with early onset insulin-dependent diabetes mellitus. Neurogenetics 7, 259-63.

17. Nannya Y, Sanada M, and Nakazaki K (2005) A robust algorithm for copy number detection using high-density oligonucleotide single nucleotide polymorphism genotyping arrays. Cancer. Res. 65, 6071-9.

18. Yamamoto G, Nannaya Y, Kato M, Sanada M, Levine R.L, Kawamata N, Hangaishi A, Kurokawa M, Chiba S, Gilliland G.D et al (2007) Highly Sensitive Method for Genomewide Detection of Alleic Composition in Nonpaired, Primary Tumor Specimens by Use of Affymetrix Single-Nucleotide-Polymorphism Genotyping Microarrays. Am. J. Hum. Genet. 81,114-126.

19. Hoffmann K and Lindner T.H (2005) EasyLINKAGE-Plus: automated linkage analyses using large-scale SNP data. Bioinformatics 21, 3565-7.

20. Bustin S.A, Benes V, Garson J.A, Hellemans J, Huggett J, Kunista M, Mueller R, NolanT, Pfaffl M.W, Shipley G.L, et al (2009) The MIQE Guidelines: Minimum Information for Publications of Quantitative Real-Time PCR Experiments. Clinical Chemistry 55, 611-622.

21. Yiu W.H, Poon J.W, Tsui S.K, Fung K.P, Waye M.M (2004) Cloning and characterization of a novel endoplasmic reticulum localized G-batch protein domain protein, IER3IP1. Gene ,377, 37-44.

22. Heidtman H, Chen C.A, Collins R.N, and Barlowe C (2005) Yos1p in a Novel subunit of the Yip1p-Yif1p Complex and IS Required for Transport between the Endoplasmic Reticulum and the Golgi Complex. Mol.Bio.Cell. 16,1673-1683.

23. Bendtsen J.D, Nielsen H, von Heijne G, and Brunako S (2004) Improved prediction of signal peptides: Signal P 3.0. J. Mol. Biol. 34,783-795.

24. Dunlavy D.M, O`Leary D.P, Kilmoy D, and Thirumalai D (2005) HOPE; A homotopy optimization method for protein structure prediction. J. Comput. Biol. 12, 1275-88.

25. Venselaar H, te Boek T.A.H, Kuippers R.K.P, Hekkelman M.L, and Vriend G (2010) Protein structure analysis of mutations causing inheritable diseases. An e-Science approach with life scientist friendly interfaces. BMC bioinformatics 11, 548.

26. Grantham R (1974) Amino acid difference formula to help explain protein evolution Science 185, 862-4.

27. Adzhubei I.A, Schmidt S, Peshin L, Ramesnsky V.E, Gerasimova A, Bork P, Kondrashov A.S, and Sunyaev S.R (2010) A method and server for predicting damaging missense mutations. Nat. Methods. 7, 248-9.

28. Ng P.C, and Henikoff S (2001) Predicting deleterious amino acid substitutions. Genome Res. 11, 863-74.

29. Bromberg Y, and Yachdav G and Rost B (2008) SNAP predicts effect of mutations on protein function. Bioinformatics 24, 2397-8.

30. Ferrer-Costa C, Gelpi J.L, Zamakola L, Parraga I, de la Cruz X, and Orozco M (2005) PMUT: a web-based tool for the annotation of pathological mutations on proteins. Bioinformatics 21,3176-8.

31. Diez-Roux G, Banfi S, Sultan M, Geffers L, Anand S, Rozado D, Magen A, Canisio E, Pagani M, Peluso I, et al (2011) A High-Resolution Anatomical Atlas of the Transcriptome in the Mouse Embryo. PLoS Biol. 9, e1000582.

32. Chang T, Wentzel E.A, Kent O.A, Ramachandran K, Mullendore M, Lee KH, Feldmann G, Yamakuchi M, Ferlito M, Lowenstein C.J et al (2007) Transactivation of miR-34a by p53 broadly influences gene expression and promotes apoptosis. Mol. Cell. 26, 745-752.

33. Boztug K, Appaswamy G, Ashikov A, Schäffer A.A, Salzer U, Diestelhorst J, Germeshausen M, Brandes G, Lee-Gosier J, Noyan F et al. (2009) A syndrome with congenital neutropenia and mutations in G6PC3. New Engl. J. Med. 360, 32-43.

34. Yiu W.H, Yeung T.L, Poon J.W.M, Tsui S.K.W, Fung K.P, and Waye M.M.Y (2010) Transcriptional regulation of IER3IP1 gene by tumor necrosis factor-alpha and Sp family proteins. Cell Biochem.Funct. 28, 31-37.

35. Sanes D.H, Reh T.A, Harris W.A.(2006) Development of the nervous system. (Elsevier Academic Press, Oxford). Second edition, pp. 173-206.

36. Kraft A.D, McPherson C.A, and Harry G.J (2009) Heterogeneity of microglia and TNF signalling as determinants for neuronal death or survival. Neurotoxicology 30,785-793.

37. Guicciardi M.E, Gores G.J (2009) Life and death by death receptors. FASEB J. 6, 1625-37.

38. Decraene C, Brugg B, Ruberg M, Eveno E, Matingou C, Tahi F, Mariani J, Auffray C, and Pietu G (2002) Identification of genes involved in ceramide-dependent neuronal apoptosis using cDNA arrays. Genome Biol. 3, 1-22.

39. Zhang Y, Scholssman S.F, Edwards R.A, Ou C.N, Gu J, and Wu M.X (2002) Impaired apoptosis, extended duration of immune responses, and a lupus-like autoimmune disease in IEX-1-transgenic mice. Proc. Natl. Acad. Sci. USA 99, 878-83.

40. Arlt A, and Schäfer H (2010) Role of the immediate early response 3 (IER3) gene in cellular stress response, inflammation and tumorigenesis. Eur. J. Cell Biol. 3, 25 November, epub ahead of print).

41. Yu T.W, Mochida G.H, Tischfield D.J, Sgaier S.K, Flores-Sarnat L, Sergi C.M, Topcu M, McDonald M.T, Barry B.T, Felie J.M et al (2010) Mutations in WDR62, encoding a centrosome-associated protein, cause microcephaly with simplified gyri and abnormal cortical architecture. Nat. Genet. 42,1017-1020.

42. Shen J, Gilmore E.C, Marshall C.A, Haddadin H, Reynolds J.J, Eyaid W, Bodell A, Barry B, Gleason D, Allen K et al (2010) Mutations in PNKP cause microcephaly, seizures and defects in DNA repair. Nat. Genet. 42, 245-9.

43. Harding H.P, Zeng H, Zhang Y, Jungries R, Chung P, Pleksen H, Sabatini D.D, and Ron D (2001) Diabetes mellitus and exocrine pancreatic dysfunction in perk-/- mice reveals a role for translational control in secretory cell survival. Mol. Cell **7**, 1153-63.

Web Resources

The URLs for data presented herein are as follows:

NCBI, geoprofile , www.ncbi.nlm.nih.gov/geoprofiles

Genepaint, http://www.genepaint.org

EMBL_EBI, http://www.ebi.ac.uk/embl

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/omim

easyLINKAGE, http://compbio.charite.de/genetik/hoffmann/easyLINKAGE/

Polyphen2, http://genetics.bwh.harvard.edu/pph2/

SIFT, http://sift.jcvi.org/

SNAP, http://rostlab.org/services/snap/

Uniprot protein sequence and functional information browser, http://www.uniprot.org

Pmut, http://mmb2.pcb.ub.es:8080/PMut

Allen Brain atlas http://www.brain-map.org/

Signal P 3.0 server http://www.cbs.dtu.dk/services/SignalP/

Chapter 3

DNA Repair and Microcephaly

Chapter 3.1

Progressive cerebellar atrophy and polyneuropathy: expanding the spectrum of PNKP mutations

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Abstract

We present a neurodegenerative disorder starting in early childhood of two brothers consisting of severe progressive polyneuropathy, severe progressive cerebellar atrophy, microcephaly, mild epilepsy and intellectual disability. The cause of this rare syndrome was found to be a homozygous mutation (c.1250_1266dup, resulting in a frameshift p.Thr424GlyfsX48) in *PNKP,* identified by applying homozygosity mapping and whole genome sequencing. Mutations in *PNKP* have previously been associated with a syndrome of primary microcephaly and seizures (MCSZ syndrome, MIM 613402), but not with a neurodegenerative disorder. PNKP is a dual function enzyme with a key role in different pathways of DNA damage repair. DNA repair disorders can result in accelerated cell death, leading to underdevelopment and neurodegeneration. In skin fibroblasts from both affected individuals we show increased susceptibility to apoptosis under stress conditions and reduced *PNKP* expression. PNKP is known to interact with DNA repair proteins involved in the onset of polyneuropathy and cerebellar degeneration, therefore our findings explain this novel phenotype.

Keywords: DNA repair, microcephaly, polyneuropathy, cerebellar atrophy, PNKP, MCSZ syndrome

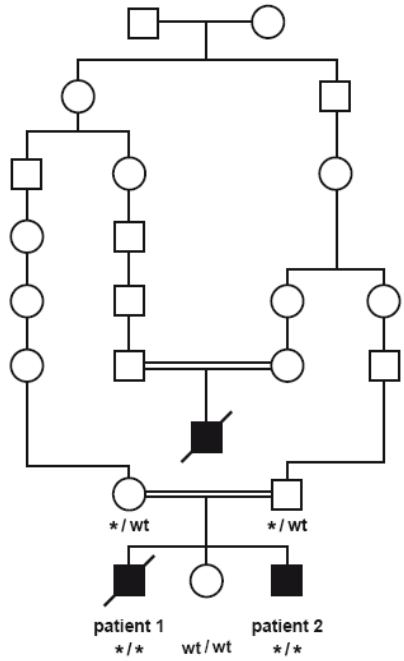
Introduction

From the first identification of the basis of mendelian disorders using whole exome sequencing (WES)[1] and whole genome sequencing (WGS)[2,3] these techniques have rapidly emerged as effective methods in finding disease-causing mutations. In consanguineous pedigrees this can be combined with homozygosity mapping for rapid candidate gene filtering[4]. Two siblings from a Dutch isolated population presented to our clinic with a rare combination of features starting in early childhood consisting of ataxia caused by progressive cerebellar atrophy, progressive debilitating polyneuropathy, microcephaly, severe intellectual disability (ID) and mild epilepsy. No ocular signs or involvement of other organs were present. No known disorder was compatible with this phenotype (including known DNA repair disorders and hereditary neuropathies), extensive metabolic work-up was normal. The MRI patterns were not typical for known cerebellar ataxia with polyneuropathy syndromes, such as infantile neuroaxonal dystrophy or neuroacanthocytosis.

Despite multiple expert opinions and laboratory studies we were unable to establish a definitive clinical diagnosis. We therefore pursued a genotype-first approach and chose a combination of homozygosity mapping and whole genome sequencing approach in order to minimize the chance of missing known and putative pathogenic mutation(s) in both coding regions and splice sites[5]. The same strategy has been successfully used to screen all known and identify new hereditary motor and sensory neuropathy (HMSN) genes[2]. To our surprise we identified a homozygous mutation in *PNKP* (MIM:605610) in both affected siblings by WGS and confirmed the disease locus with homozygosity mapping. Mutations in *PNKP* (polynucleotide kinase 3`-phosphatase) have been described in an autosomal recessive disorder characterized by microcephaly, seizures and developmental delay (MCSZ, one of the early infantile epileptic encephalopathies, EIEE10, MIM: 613402)[6]. The MCSZ patients are phenotypically very different to the patients described, who did not show any clinical progression, or any neurodegenerative symptoms. Additional studies in patient cells indicate a role of increased apoptosis in the pathogenesis of this novel degenerative phenotype.

Clinical Reports

The parents of our patients are healthy and originate from an isolated community in the southwest of the Netherlands. Genealogical studies of the family pedigree showed a loop of consanguinity (Figure 1A).



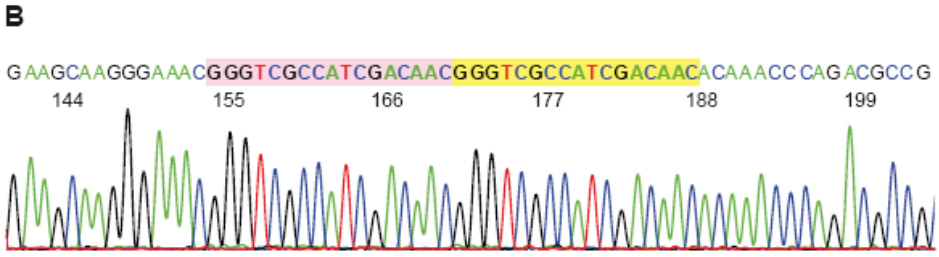


Figure 1. A: Pedigree of the family showing common ancestor couple and the link to a possibly third affected family member. *PNKP* genotype is shown below individuals: *= 1250_1266 dup, wt= "wild type" *PNKP* sequence, black boxes represent affected persons. B: Sanger sequencing results of *PNKP* in patient 2, the sequence present *in duplo* is shaded in pink and yellow.

Patient 1 was born at term with microcephaly (occipito-frontal circumference, OFC -3.25 SD), and weight and length at -1 SD. He had developmental delay, at 15 months he was able to take a few supported steps, and say 'daddy' and 'mama'. At this age his OFC was -5.7 SD. At 23 months he walked independently, with a broad based ataxic gait. He had brisk tendon reflexes. He presented with a series of febrile seizures at 2.5 years, and experienced occasional seizures, mostly febrile, thereafter. His global development was compatible with 17 months at age 4.5 years (Bayley scale of development). The boy was hyperactive and always had

an extreme happy behaviour. The diagnosis Angelman syndrome was considered, but DNA testing was negative. At age 9 he was a restless boy with severe progressive microcephaly (-6 SD), short stature (-4 SD), ataxic gait, speaking 5 to 10 words. A brain MRI at age 16 showed severe cerebellar atrophy (Figure 2, A-C), while a CT made at the age of 10 months showed no atrophy of the cerebellum at all.

A slow deterioration of motor skills became evident, and he became wheelchair bound at age thirteen (see supplemental video). Motor function further deteriorated over the next few years, mostly as the consequence of progressive signs of the peripheral neuropathy, i.e. paresis of arms and legs accompanied by hypotonia, muscular atrophy, loss of the initially brisk tendon reflexes, and the development of contractures. A progressive severe motor and sensory demyelinating polyneuropathy with axonal degeneration was shown on EMG at age 11 and 16 (Table 1). In contrast to the motor deterioration, cognitive functions and speech performance remained stable, while the epilepsy improved. He retained a happy demeanour, but had temper tantrums in adolescence. He died of pneumonia at 25 years.

Patient 2, the younger brother of patient 1, was born at term with birth weight at +0.5 SD, length at -0.25 SD, and OFC at -1.5 SD. At 3 months microcephaly (-3.5 SD) and developmental delay were noted. His first seizure occurred at 12 months. He walked independently at 18 months and showed also hyperactive and happy behaviour. At five years he developed strabismus and papilloedema consistent with raised intracranial pressure and a ventriculo-peritoneal shunt was placed. He slowly lost his ability to walk, and started using a wheel chair from age 7 (see supplemental video). At age 14 his OFC was -4.75 SD, and he was a hyperactive boy who could speak about 10 words. EMG at age 10 and 16 showed demyelinating polyneuropathy (Table 1). Brain MRI at age 15 months showed enlarged lateral ventricles and thin corpus callosum (Figure 2, D-F), and at age 9 and 18 years, after placement of the VP drain, showed a severe progressive cerebellar atrophy, and mild cerebral atrophy (Figure 2, G-L). Seizures occurred 3-4 times a year while on carbamazepine therapy, but later on the frequency decreased. No eye problems, skin rash or susceptibility to infection were ever observed in the brothers.

A second-degree cousin of the father (Figure 1A) had a similarly affected son who died around 30 years of age of a progressive neurological disease. No medical records are available. He had a small head, seizures and had lost the ability to walk.

Metabolic investigations including lactate, vitamin A and E, very long chain fatty acids, sialotransferrin isoforms, erythrocyte and leukocyte morphology, lysosomal enzymes, urine organic acids, mucopolysaccharides and amino acids, and CSF neurotransmitters were normal in both brothers. Transcription-coupled nucleotide excision repair after UV treatment (diagnostic test for Cockayne syndrome) and inhibition of DNA synthesis after X-ray irradiation (diagnostic test for ataxia-telangiectasia) were normal in patient fibroblasts (see below).

A video of patient 1 and 2 at different ages showing ataxia and progressive deterioration is included in the supplemental data (with consent of the family).

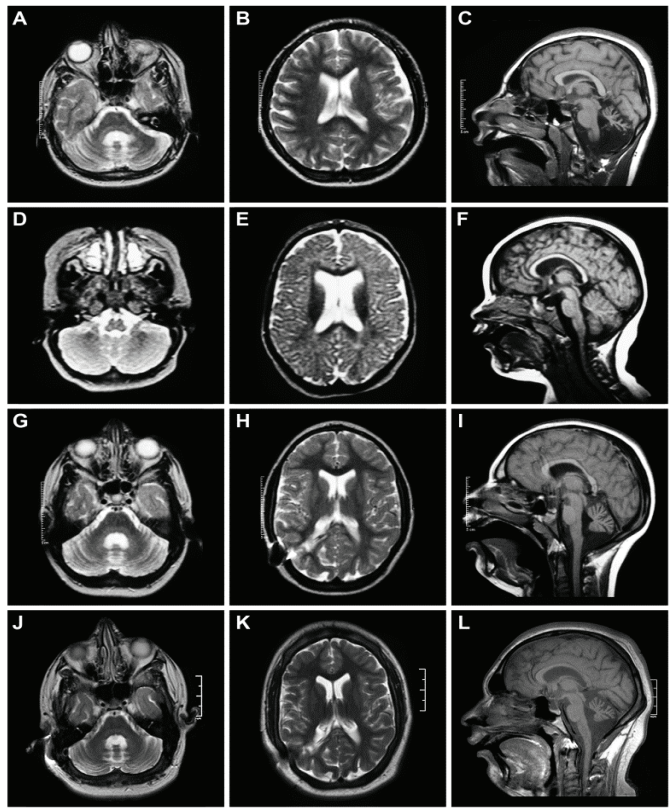


Figure 2. MRI imaging of patient 1 (A-C) and patient 2 (D-L). First two columns show transversal T2-weighed images, the third column shows sagittal T1-weighed images. A-C: patient 1 age 16 years, microcephaly, generalized atrophy, enlarged ventricles, and severe cerebellar atrophy. D-F: patient 2 age 15 months, mild generalized atrophy, enlarged lateral ventricles and thin corpus callosum. G-I: age 9 years, and J-L: age 18 years, show decrease of lateral ventricle sizes after placement of VP-drain, severe and progressive cerebellar atrophy.

Materials and Methods

Patient material and informed consent

All the family members and legal patient guardians provided written informed consent for participation in the study, which is embedded in a broader study of the cause of brain malformation at the Erasmus MC.

SNP Arrays

500 ng of total genomic DNA extracted from peripheral leucocytes of the two affected brothers and their unaffected sister was hybridized to Genome-Wide Human SNP Array 6.0 according to the manufacturer's protocol (Affymetrix, Santa Clara, CA). Affymetrix Genotyping Console (GTC4.1.0) was used for genotyping and both GTC4.1.0 and CNAG v3.3.0.1 Beta were used for regions of homozygosity (ROH) analysis as well as copy number analysis[7].

Whole Genome Sequencing (WGS)

DNA from the mother and two affected sons was extracted from peripheral leucocytes (mother) and fibroblast cell lines (sons) and sent to Complete Genomics (Mountain View, US), for whole genome sequencing[8]. This technique was preferred above exome sequencing in order to minimize the chance of missing the mutations by exon capturing techniques[9].

Reads were mapped to the National Centre for Biotechnology Information (NCBI) reference genome, build 36. Analysis of the whole genome sequencing data was done using CGA tools version 1.3.0 and TIBCO Spotfire version 3.3.1. The initial analysis was restricted to nonsynonymous variants, variants disrupting the acceptor or donor splice site or small insertions or deletions (up to approximately 50 bp) in the homozygous area that were fully called and were not present in dbSNP129, or in data from the 1000 genome database and Exome variant sever (Washington university), or in 100 control samples from our in-house database (HuVariome). Additionally, variants should follow the expected autosomal homozygous recessive inheritance pattern. When this analysis did not lead to a result, the stringency of the filtering was lowered by allowing no-calls or half-calls at potential variant positions, as long as one of the family members showed a variant on at least one allele. As a compound heterozygous recessive or X-linked inheritance could not be excluded by the pedigree of this family, we also performed analyses for these inheritance models.

Sanger Sequencing

Verification of the mutation was performed by Sanger sequencing of exon 14 of the *PNKP* gene (RefSeq NG_027717) on DNA isolated from peripheral leucocytes. PCR amplification reactions were performed and products were purified with ExoSAP-IT (USB). Direct sequencing of both strands was performed using Big Dye Terminator chemistry ver. 3.1 (Applied Biosystems). DNA fragment analysis was performed using capillary electrophoresis on an ABI 3130 Genetic Analyzer (Applied Biosystems) and the software package Seqscape (Applied Biosystems, version 2.1). Primer sequences are available upon request.

DNA repair and Apoptosis Studies

In cultured skin fibroblasts from patient 1 and 2, transcription-coupled nucleotide excision repair after UV treatment was used as diagnostic test for Xeroderma pigmentosum and Cockayne syndrome as described and resistance to inhibition of DNA synthesis after X-ray irradiation (cell cycle arrest) was used as a diagnostic test for mutations in the ATM pathway (ataxia-telangiectasia and Nijmegen breakage syndrome)[10,11,12].

Using cultured skin fibroblasts from both patients bearing the homozygous *PNKP* mutation, and 5 healthy control cells lines, we tested for susceptibility to apoptosis. In order to detect apoptosis the cells were stained with the FlicaTM (Fluorescent-Labeled Inhibitor of Caspases) apoptosis multi-caspase detection kit (Immunochemistry Technologies, LLC), which makes use of an inhibitor sequence of active caspases and was performed according to the manufacturer instructions. Fluorescent cells were scored (as a percentage of apoptotic fibroblasts) before and after stress induction by 24 hours serum deprivation and then a further 24 hours exposure to 5 mM dithiothreitol (DTT) by two blinded investigators. Necrosis versus apoptosis was tested by vital staining exclusion with propidium iodide. The experiment was repeated three times and each time performed in triplicate.

Quantitative PCR (qRT-PCR)

Analysis of gene expression by qRT-PCR was carried out using a KAPA SYBRH FAST qPCR Kit (Kapa Biosystems) in the CFX96 Real-Time system (BioRad). Thermal cycling conditions were as follows: denaturing step (95˚C for 3 minutes), followed by 35 cycles of denaturing (95˚C for 5”), annealing and extension (60˚C for 30”). Fluorescence detection and

data analysis were performed by BioRad CFX Manager 2.0. Experiments were performed in triplicate using as the reference *GAPDH* (MIM 138400) and *ACTB* (MIM 102630) for gene expression normalization[13]. Primers are listed in supplemental Table S3.

Results

SNP arrays and WGS

ROH analysis of the two affected brothers and an unaffected sister was performed in CNAG 3.3.0.1 Beta. Two regions of overlapping ROH were found in the two affected brothers and not in the unaffected sister on chromosome 11q23 (bp 117,449,498-118,276,116, build 37) and chromosome 19q13 (bp 34,230,986-51,258,314, build 37). Considering the consanguinity loop and the size of the area (17 Mb), the region on chromosome 19q13 can be confidently considered identical by descent rather than identical by state and would putatively contain the genetic mutation (Figure S1). The mapped whole genome sequence of the three samples (mother and two sons) varied between 183 and 192 Gb, resulting in a good average coverage between 66x and 68x per genome (supplemental Table S1). Confident diploid calls could be made for approximately 97% of the reference genome in all cases. On average, 3.75 million genetic variants were identified per sample, including single nucleotide variants and short insertions, deletions and substitutions up to ~50 bp.

Filtering for a homozygote recessive mutation present in both affected individuals was applied and after allowing no calls, variations in only 3 genes in the homozygous regions were found: *ZNF285A*, *FCGRT* and *PNKP* (Figure S2). We excluded FCGRT as this encodes a protein only expressed in placental tissue and responsible for transferring IgG from mother to fetus therefore is not relevant to the phenotype. *ZNF285A* is 100% identical to human gene *ZNF806* and *ZNF678*, it has no mouse ortholog which throws doubt onto the physiological mechanism, and is in a cluster of more then 25 similar ZNF genes on chromosome 19. This makes it unlikely that this is the causative gene for this phenotype.

The *PNKP* variant was confirmed by Sanger sequencing, which showed an insertion of 17 bp corresponding to a duplication in exon 14 of PNKP (c.1250_1266dup) resulting in a frameshift (p.Thr424GlyfsX48, PNKP-201 Ensembl database). This insertion is within the largest candidate ROH (supplemental Figure S1 and Table S1). The *PNKP* gene was recently described in literature to be related to microcephaly, seizures and

developmental delay (MCSZ) syndrome and one of the mutations was the same variant that we identified in our family[6]. Although heterozygous for the duplication, the mother was homozygous for the haplotype previously described. This confirms the association of the duplication with this haplotype in different ethnic backgrounds, and suggests this haplotype might predispose to the occurrence of the duplication, instead of being a founder effect. Analysis of WGS data for compound heterozygous and X-linked inheritance did not lead to any additional causative variants being found. We also specifically looked into genes known to be involved in neuropathy (Table S4) and found sufficient coverage and sequencing depth to confidently exclude a causative mutation.

qRT-PCR

In order to confirm the 17 bp duplication in exon 14 and to study its effect on *PNKP* expression we studied transcripts by qRT-PCR. Using primers against *PNKP* and designed over exon 3 and 4 and exon 15 and 16 we showed that the expression of PNKP was consistently decreased, however there was still expression of mRNA for PNKP in the homozygous state (supplemental Figure S3). These results support the effect of the mutation as a loss of PNKP function.

Apoptosis Studies

One of the effects of DNA damage is that, due to overall cell stress, cells have an increased susceptibility to undergo apoptosis. It has also been hypothesized that abnormal apoptosis is the pathogenic mechanism leading to microcephaly in MCSZ[6]. Cultured skin fibroblasts from both patients had indeed a significantly increased tendency to undergo apoptosis (Student's unpaired *t*-test $p<0.0001$) when under stress conditions in comparison to 5 control cell lines (Figure 3).

Discussion

We successfully applied WGS to identify the disease causing *PNKP* mutations in two brothers with a novel phenotype consisting of progressive cerebellar atrophy, severe polyneuropathy, microcephaly, and severe ID. There were occasional seizures, but no history of early infantile epileptic encephalopathy.

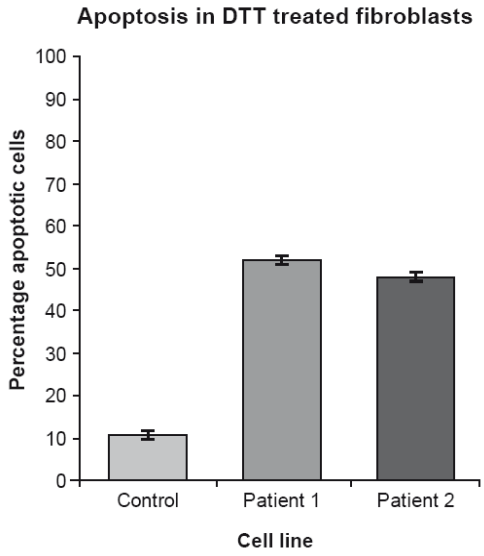


Figure 3. Patient 1 and patient 2 fibroblasts have an increased tendency to undergo apoptosis when treated with 5mM DTT in comparison to 5 control cell lines, each experiment involved testing 5 control cell lines and 2 patient cell lines on 3 separate occasions. ($p<0.001$, unpaired *t*-test, SPSS v17.0). Rates are shown as a mean of 3 experiments (+/- SEM).

The same 17 bp duplication in exon 14 (c.1250_1266dup, resulting in a frame shift Thr424GlyfsX48) in *PNKP* in homozygous and compound heterozygous state has been identified in patients with a different syndrome (MCSZ/EIEE10, microcephaly, intractable seizures and developmental delay syndrome)[6]. The published three patients who were homozygous for this mutation within an identical haplotype were not described as having progressive neurodegeneration. However, our patients clearly showed progressive disease in childhood, with loss of motor milestones (see supplemental video), development of severe neuropathy and cerebellar atrophy. The MCSZ syndrome was not considered in our patients because of these symptoms. The extensive diagnostic investigations, the combined autozygosity mapping and WGS approach makes very unlikely that in this family we missed another genetic mutation accounting for the disorder.

PNKP is a dual function protein that has a polynucleotide 3'phosphatase and a polynucleotide 5` hydroxylkinase domain and has an important role in repair of both single strand breaks (SSB) and double-strand breaks (DSB)[14,15]. Fibroblast cell lines from our PNKP patients showed an increased tendency to go into apoptosis, supporting a role of apoptosis

in the pathogenesis. Our observations suggest that stress placed on the cell by the DNA repair defect causes insufficient proliferation and cell death. Increased apoptosis was shown in mouse neuronal precursors and differentiated neurons with reduced *Pnkp* levels[6].

We looked at PNKP's interacting partners using STRING analysis, which combines information from different sources to identify known and putative protein interactions (Figure 4). This shows interaction with known neurodegenerative disease-associated proteins (Figure 4). Mutations

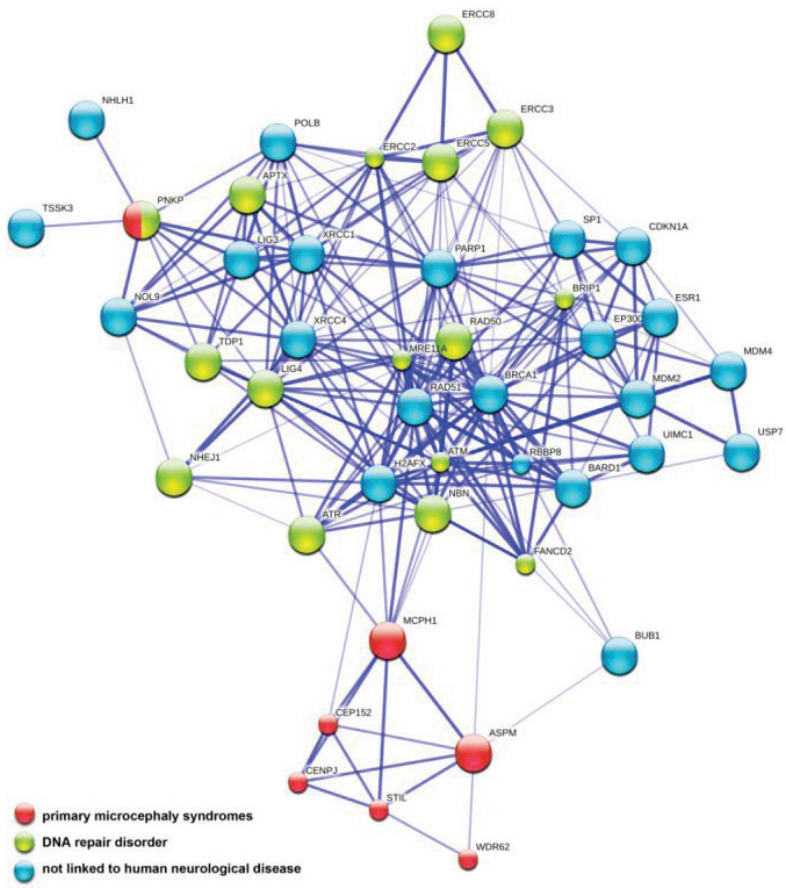


Figure 4. STRING analysis showing PNKP interactions between known proteins which are encoded by genes associated with primary microcephaly syndromes (red filled circles), DNA repair disorders (green filled circles), proteins not yet associated with human neurological disease (candidate genes, blue filled circles). The blue line between the proteins indicate known interactions and the thickness of the line is representative of the confidence of evidence (the thicker the line, the more evidence to support the interaction).

in *TDP1* cause autosomal recessive spinocerebellar ataxia with axonal neuropathy (SCAN1, MIM 607250)[16], and this has led to the previous suggestion that *PNKP* could be a gene for SCAN[17]. *APTX* mutations cause ataxia-oculomotor apraxia type 1 (AOA1, MIM 606350). *PNKP*, *TDP1*, and *APTX* defects all lead to abnormal SSB repair, which provides experimental evidence for a common mechanism of neurodegeneration[18].

Severe microcephaly is observed in the autosomal recessive primary microcephaly syndromes (MCPH). One of the genes involved in this disorder, MCPH1, links the MCPH genes to DNA repair by activating ATM and ATR (respectively associated with ataxia-telangiectasia and Seckel syndrome)[14,19], which in turn phosphorylate and activate PNKP.

In the DSB repair, which is particularly important in dividing cells during development, PNKP directly interacts with the scaffold XRCC4 and activates LIG4. *LIG4* mutations cause immunodeficiency with microcephaly (MIM 606593)[19,20]. *DNA ligase 4 -/-* mice show increased apoptosis throughout the nervous system and die in the embryonic period. *Lig4* conditional KO mice (*Lig4 Nes-Cre*) have a pronounced microcephaly and show increased apoptosis from E13.5, peaking at E15[21]. This apoptosis is apparently mediated by the intact pro-apoptotic function of ATM in *Lig4* mutants[22]. Microcephaly due to increased apoptosis of neuroprogenitors during embryonic development has been shown by our group in a syndrome with congenital microcephaly and diabetes[23].

Therefore, the function and interactions of PNKP supports a role in the neurodegenerative phenotype as well as microcephaly, possibly by increased apoptosis.

In conclusion, we have described a neurodegenerative phenotype not previously described with *PNKP* mutations and used a combined approach of autozygosity mapping and whole genome sequencing as a valuable tool for diagnosis.

Acknowledgements

We thank Dr N.G. Jaspers at the department of Genetics, Erasmus Medical Center for critically reading the manuscript. We acknowledge the collaboration of the members of the patients' family. We thank J. Meulstee for help with interpretation of EMG data. We also thank Tom de Vries Lentsch for the figures and Petra Veraart for the genealogical study.

Table 1. Nerve conduction velocities and EMG results of both patients.

EMG Patient 1 (11 years)	**Motor nerve**	**Muscle**	**DML**	**NCV**	**SNAP**	**CMAP: distal stimulation**	**CMAP: proximal stimulation**
	R ant tib	EDB	10.4 ms	72 m/s		0.4 mV	0.6 mV
	R median	APB	3.5 ms	41 m/s		5.0 mV	4.5 mV
EMG Patient 1 (16 years)	R tib ant	EDB				0 mV	
	R post tib	AH				0 mV	
	L post tib	AH				0 mV	
	R median	APB	5.3	34 m/s		4.5 mV	2.1 mV
	Sensory nerve						
	L sural				0 uV		
	R median (digit 3)				0 uV		
	Needle EMG		FP	PSW	Maximal pattern	Polyphasic MUAP	
		L TA	+	+	0		
		L Q	+	-	mixed	+	
EMG Patient 2 (11 years)	Motor nerve	Muscle	DML	NCV	SNAP	CMAP: distal stimulation	CMAP: proximal stimulation

	R ant tib	EDB				0 mV	0 mV
	R post tib	AH	6.5 ms	34 m/s		2.8 mV	1.5 mV
	L post tib	AH	4.9 ms	37 m/s		2.7 mV	2.4 mV
	Sensory nerve						
	L sural nerve				0 uV		
	Needle EMG		FP	PSW	Maximal pattern	Polyphasic MUAP	
		L tib ant	-	-	mixed	-	
EMG Patient 2 (16 years)	Motor nerve	Muscle	DML	NCV	SNAP	CMAP: distal stimulation	CMAP: proximal stimulation
	R ulnar nerve	ADM	4.4 ms	31		0.7 mV	0.8 mV
	R post tib	Soleus					0 mV
	L post tib	AH				0 mV	
	Sensory nerve						
	R sural nerve				0 uV		
	R median (digit 3)				0 uV		
	Needle EMG		FP	PSW	Maximal pattern	Polyfasic MUAP	
		R TA	++	-	0		
		R FR	++	+++	0		
		R BB	-	-	mixed	+	

Table 1. Nerve Conduction Velocity (NCV) and Distal Motor Latency (DML) values for patients 1 and 2 show slowing and loss of conduction in motor and sensory nerves, conduction block and denervation, consistent with a severe mixed demyelinating/axonal sensorimotor polyneuropathy. Normal values: NCV > 50 m/s in the arms, > 40 m/s in the legs; Compound Motor Action Potential (CMAP), Extensor Digitorum Brevis (EDB) > 5 mV; CMAP muscles hand > 10 mV.

Additional abbreviations used in the table: Abductor Digiti Minimi (ADM); Abductor Hallucis (AH); Abductor Pollicis Brevis (APB); Biceps Brachii (BB); Femoris Rectus (FR); Fibrillations (FP); Motor Unit Action Potentials (MUAP); Positive Sharp Waves (PSW); Quadriceps (Q); Sensory Nerve Action Potential (SNAP);Tibialis Anterior (TA). Anterior tibial nerve (ant tib); posterior tibial nerve (post tib). Right Side (R); Left Side (L)

Supplemental data

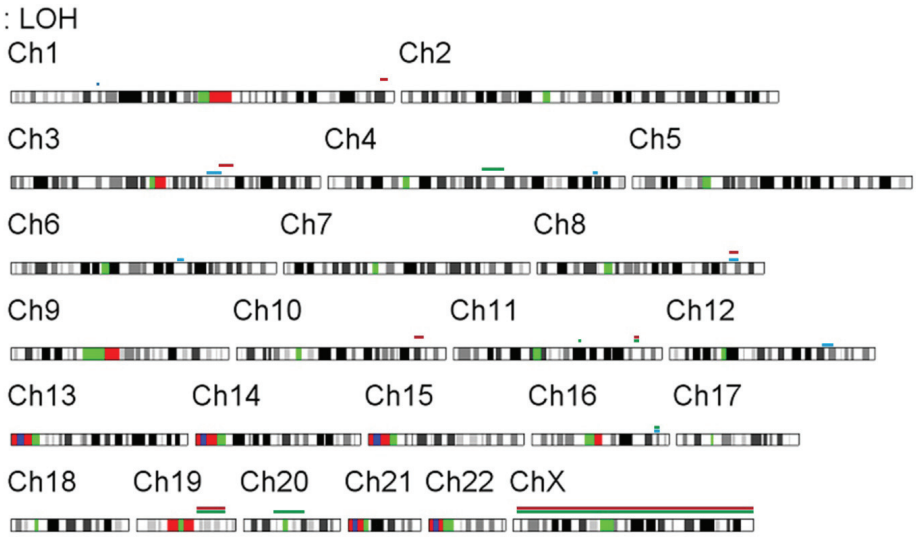


Figure S1. CNAG view showing overlapping regions of homozygosity (ROH) on chromosome 11 and 19 among the three sibs (two affected brothers and one unaffected sister). The blue bar represents ROH of the unaffected sister, the red and green bars represent the brothers (i.e. the overlapping ROH on chromosome 8 concern the unaffected sister and one affected brother).The brothers share ROH on chromosome 11 and 19.

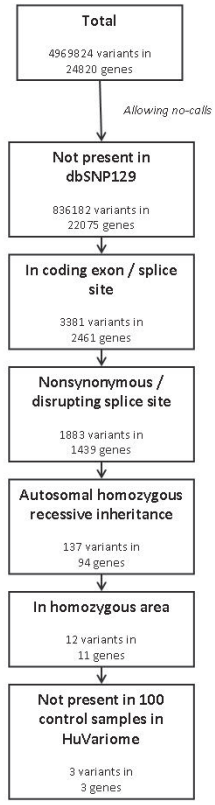


Figure S2. Whole genome sequence analysis. Figure showing filtering steps starting from the list of variants found and leading to candidate genes.

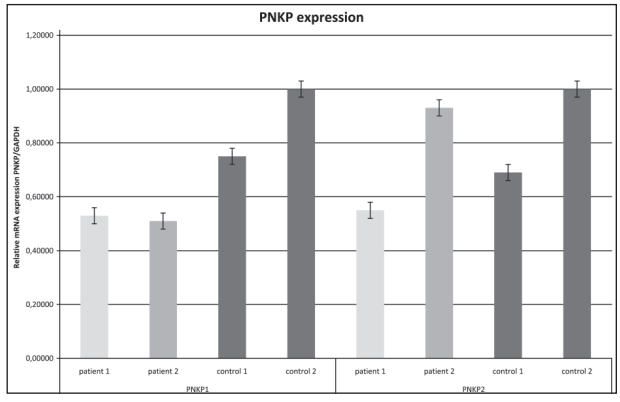


Figure S3. *PNKP* mRNA expression. qRT-PCR data of *PNKP* relative to the housekeeping gene *GAPDH* in fibroblasts from patient 1 and 2 and two controls, using two different primers (PNKP1, covering exons 3-4, and PNKP2, covering exons 15-16) showing variable, but not absent expression in fibroblasts.

Table S1. Whole genome data analysis. Sequencing statistics for the three samples.

Patient info	Gender	Average coverage	Called genome fraction		SNP transitions/ transversions	SNPs		Insertions		Deletions		Substitutions	
			fully	*partially*		*total*	*novel rate*	*total*	*novel rate*	*total*	*novel rate*	*total*	*novel rate*
Mother	female	67x	0.968	0.005	2.13	3341703	0.066	192689	0.209	211083	0.239	72368	0.344
Patient2	male	68x	0.967	0.005	2.14	3262278	0.066	186261	0.212	201744	0.237	69509	0.343
Patient1	male	66x	0.965	0.006	2.14	3283423	0.065	183554	0.210	199672	0.238	69792	0.339

Table S2. List of genes extrapolated from the STRING analysis, coding for proteins involved in DNA repair defects and primary microcephaly and proven to interact directly or indirectly. Specification of the gene function and the association with (human) disorders is illustrated here.

Symbol	OMIM	Name	Function	Disease
PNKP	605610	Polynucleotide Kinase 3-phosphatase	Catalyses 5-prime phosphorylation of nucleic acids amd 3-prime phosphatise activity-DNA repair	Epileptic Encephalopathy, early infantile
TDP1	607198	Tyrosyl-DNA phosphodiesterase 1	Catalyses the hydrolysis of the phosphodiester bond between tyrosine residue and DNA 3 prime phosphate	Spinocerebellar ataxia with axonal neuropathy
XRCC1	194360	x-ray repair, comple-menting defective in Chinese hamster,1	Forms a complex with PNK, POLB and LIG3 repair single strand DNA breaks	
POLB	174760	DNA polymerase B	Carries out base excision repair (BER)	
XRCC4	194363	X-ray repair, complementing defective in Chinese hamster	Restores DNA double strand break repair and supports V(D)J recombination	

LIG4	601837	Ligase IV,DNA, ATP dependent	Joins single strand breaks in a double stranded polydeoxynucleotide in an ATP dependent reaction Responsible for ligation step in nonhomologous DNA end joining and V(D)J recombination	LIG4 syndrome – microcephaly, growth retardation, pancytopenia, myelopdysplasia, chronic resp infections, photosensitivity, telangiectasia, hypothyroidism and type II diabetes
LIG3	600940	Ligase III, DNA, ATP dependent	Mitochondrial dependent repair	
NOL9		Nucleolar protein 9	Polynucleotide 5`kinase involved in ribosomal RNA processing	
NHLH1	162360	Nescient Helix Loop Helix 1		
TSSK3	607660	Testis specific serine kinase 3	Thought to play a role in either germ cell differentiation or mature sperm function	
NHEJ1	611291	Non homologous end joining factor 1	SCID associated with microcephaly and growth retardation	
PARP1	173870	Poly(ADP-Ribose) Polymerase 1	Role in repair of ss DNA breaks	?mental retardation
SP1	189906	Transcription factor specificity protein 1	Activate or repress transcription in response to wide range of stimuli	
CDKN1A	116899	Cyclin dependent kinase inhibitor 1A	Mediates cell cycle arrest in response to the p53 checkpoint pathway	
EP300	602700	E1A-Binding protein, 300 KD	Encodes a histone acetyltrasnferase that regulates transcription via chromatin remodelling and is important in the process of cell proliferation and differentiation	Colorectal Cancer Rubenstein Taybi Syndrome 2
MDM4	602704	Mouse Double Minute 4 Homolog	Critical regulator of p53	
MDM2	164785	Mouse Double Minute 2 Homolog	Major regulator of tumour suppressor p53 by targeting its destruction	

ESR1	133430	Estrogen receptor 1	Ligand activated transcription factor composed of several domains important for hormone binding, DNA binding and activation of transcription	Polymorphisms associated with bone mineral density , migrane, breast cancer, MI
USP7	602519	Ubiquitin Specific Protease 7	Ubiquitin specific protease that cleaves ubiquitin from its substrates, direct antagonist of MDM2	
ATM	607585	Ataxia Telengiectasia Mutated Gene	Member of phosphatidylinositol 3 kinase family that respond to DNA repair and/or cycle control	Ataxia Telangiectasia
FANCD2	613984	Fanconi anemia complementation group D2	Involved in repair of DNA ds breaks	Fanconi anaemia
UIMC1	609443	Ubiquitin interaction motif-containing protein 1	Ubiquitin binding protein plays a central role in BRCA1-complex	
BRCA1	113705	Breast cancer 1 gene	Critical role in DNA repair, cell cycle check point control and maintenance of genomic stability	Breast-ovarian cancer familial 1 Susceptibility to pancreatic cancer
BARD1	601593	BRCA1 associated ring domain 1	Interacts with the N-terminal region of BRCA1	Susceptibily to breast cancer
BUB1	602452	Budding uninhibited by benzimadazoles 1	Required for function of spindle assembly checkpoint	Susceptibility to colorectal cancer with chromosomal instability
RBBP8	604124	Retinoblastoma binding protein 8	Co-operates with MRN complex in processing meiotic and mitotic ds breaks by ensuring both resection and intrachromosomal association of the broken ends	
ASPM	605481	Abnormal spindle like microcephaly associated	Essential role in normal mitotic spindle function	Autosomal recessive microcephaly 5
WDR62	613583	Wd repeat containing protein 62	Thought to be a centrosomal and nuclear protein	Autosomal recessive microcephaly 2
CEP152	613529	Centrosomal protein, 152KD	Core protein of centrosome with crucial function in cell division	Autosomal recessive micrcocephaly type 3 Seckel Syndrome 5

CENPJ	609279	Centromeric protein J	Encodes a centrosomal protein with a putative function in regulation of microtubule assembly and nucleation	Autosomal recessive microcephaly type 4 Seckel Syndrome 4
STIL	181590	SCL/TAL1 interrupting locus	Encodes a cytoplasmic protein implicated in regulation of mitotic spindle checkpoint	Automoal recessive microcephaly type 7
MCPH1	607117	Microcephalin	Encodes a regulator of chromosome condensation	Autosomal recessive microcephaly type 1
BRIP1	605882	BRCA1-interacting protein 1	Interacts with BRCA1 to form a complex important in ds DNA repair	Early onset breast cancer Fanconi anaemia type 1
ATR	601215	Ataxia Telangiectasia and RAD3 related	Serine/threonine protein kinase which activates checkpoint signalling upon gentoxic stresses	Seckel Syndrome 1
RAD51	179617		Mediates homologous pairing and strand exchange in recombinatory structures to provide a critical role in genomic integrity	Susceptibilty to breast cancer
H2AFX	601772	H2A Histone Family member X	Involved in nucleosomal organisation of chromatin	
ERCC2	126340	Excision repair, complementing defective in Chinese hamster 2	Encodes a ATP-dependent 5`3` DNA helicase involved in transcription coupled NER	Cerebrooculofacioskeletal syndrome 2 Trichothiodystrophy Xeroderma pigmentosa group D
ERCC3	133510	Excision repair, complementing defective in Chinese hamster 3	ATP dependent DNA helicase functions in nucleotide excision repair	Trichothiodystropy Xeroderma pigmentosum group B
ERCC5	133530	Excision repair, complementing in Chinese hamster 5	Single stranded structure specific DNA endonuclease involved in DNA excision repair	Cerebrooculofacial syndrome type 3 Xeroderma pigmentosum group G
ERCC8	609412	Excision repair cross complementing group 8	Substrate recognition component of the CSA complex involved in transcription coupled nucleotide excision repair	Cockayne Syndrome type A
APTX	606350	Aprataxin	DNA binding protein involved in ss DNA repair, ds DNA repair and BER.	Ataxia, early onset with oculomotor apraxia and hypoalbuminaemia, Coenzyme10 deficiency

Table S3. Primers used for qRT-PCR experiments of PNKP.

PNKP_qRT_1 forward	GGAGCTGGTCGCAGATCCT
PNKP_qRT_1 reverse	CCAACCCCGGCTTCAACT
PNKP_qRT_2 forward	GGCGCGCCACAACAAC
PNKP_qRT_2 reverse	CGAACTGCTTCCTGTAGCCATA

Table S4. Genes with OMIM entry which are associated with hereditary neuropathy. The WGS sequence data were specifically checked for sufficient coverage and data quality for these genes; no mutations were identified despite high quality data.

Gene	OMIM #
GDAP1	606598
MTMR2	603557
SBF2	607697
SH3TC2	608206
NDRG1	605262
EGR2	129010
PRX	605725
FGD4	611104
FIG4	609390
MED25	610197
LMNA	150330
PMP22	601097
SLC12A6	604878

References

1. Hoischen A, van Bon BW, Gilissen C, et al (2010) De novo mutations of SETBP1 cause Schinzel-Giedion syndrome. Nat Genet 42: 483-485.

2. Lupski JR, Reid JG, Gonzaga-Jauregui C, et al (2010) Whole-genome sequencing in a patient with Charcot-Marie-Tooth neuropathy. N Engl J Med 362:1181-1191.

3. Roach JC, Glusman G, Smit AF, et al (2010) Analysis of genetic inheritance in a family quartet by whole-genome sequencing. Science 328:636-639.

4. Pippucci T, Benelli M, Magi A, et al (2011) EX-HOM (EXome HOMozygosity): a proof of principle. Hum Hered 72:45-53.

5. Dixon-Salazar TJ, Silhavy JL, Udpa N, et al (2012) Exome sequencing can improve diagnosis and alter patient management. Sci Transl Med 4:138ra178.

6. Shen J, Gilmore EC, Marshall CA, et al (2010) Mutations in PNKP cause microcephaly, seizures and defects in DNA repair. Nat Genet 42:245-249.

7. Nannya Y, Sanada M, Nakazaki K, et al (2005) A robust algorithm for copy number detection using high-density oligonucleotide single nucleotide polymorphism genotyping arrays. Cancer Res 65:6071-6079.

8. Drmanac R, Sparks AB, Callow MJ, et al (2010) Human genome sequencing using unchained base reads on self-assembling DNA nanoarrays. Science 327:78-81.

9. Lam HY, Clark MJ, Chen R, et al (2012) Performance comparison of whole-genome sequencing platforms. Nat Biotechnol 30:562.

10. Kleijer WJ, van der Kraan M, Los FJ, Jaspers NG (1994) Prenatal diagnosis of ataxia-telangiectasia and Nijmegen Breakage syndrome by the assay of radioresistant DNA synthesis. Int J Radiat Biol 66:S167-174.

11. Mallery DL, Tanganelli B, Colella S, et al (1998) Molecular analysis of mutations in the CSB (ERCC6) gene in patients with Cockayne syndrome. Am J Hum Genet 62:77-85.

12. Zafeiriou DI, Thorel F, Andreou A, et al (2001) Xeroderma pigmentosum group G with severe neurological involvement and features of Cockayne syndrome in infancy. Pediatr Res 49:407-412.

13. Bustin SA, Benes V, Garson JA, et al (2009) The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem 55:611-622.

14. Caldecott KW (2008) Single-strand break repair and genetic disease. Nat Rev Genet 9:619-631.

15. Weinfeld M, Mani RS, Abdou I, Aceytuno RD, Glover JN (2011) Tidying up loose ends: the role of polynucleotide kinase/phosphatase in DNA strand break repair. Trends Biochem Sci 36:262-271.

16. Sano Y, Date H, Igarashi S, et al (2004) Aprataxin, the causative protein for EAOH is a nuclear protein with a potential role as a DNA repair protein. Ann Neurol 55:241-249.

17. Plo I, Liao ZY, Barcelo JM, Kohlhagen G, Caldecott KW, Weinfeld M, Pommier Y (2003) Association of XRCC1 and tyrosyl DNA phosphodiesterase (Tdp1) for the repair of topoisomerase I-mediated DNA lesions. DNA Repair (Amst) 2:1087-1100.

18. Reynolds JJ, Walker AK, Gilmore EC, Walsh CA, Caldecott KW (2012) Impact of PNKP mutations associated with microcephaly, seizures and developmental delay on enzyme activity and DNA strand break repair. Nucleic Acids Res.

19. McKinnon PJ (2009) DNA repair deficiency and neurological disease. Nat Rev Neurosci 10:100-112.

20. Mani RS, Yu Y, Fang S, et al (2010) Dual modes of interaction between XRCC4 and polynucleotide kinase/phosphatase: implications for nonhomologous end joining. J Biol Chem 285:37619-37629.

21. Gatz SA, Ju L, Gruber R, et al (2011) Requirement for DNA ligase IV during embryonic neuronal development. J Neurosci 31: 10088-10100.

22. Shull ER, Lee Y, Nakane H, et al (2009) Differential DNA damage signaling accounts for distinct neural apoptotic responses in ATLD and NBS. Genes Dev 23:171-180.

23. Poulton CJ, Schot R, Kia SK, et al (2011) Microcephaly with simplified gyration, epilepsy, and infantile diabetes linked to inappropriate apoptosis of neural progenitors. Am J Hum Genet 89:265-276

Web Resources

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/omim.

cga tools: http://www.completegenomics.com/sequence-data/cgatools/

TIBCO Spotfire: http://spotfire.tibco.com/

STRING 9.0 http://string-db.org.

www.ensembl.org

Chapter 3.2

Severe microcephalic Cerebro-Ocular-Facio-Skeletal (COFS) syndrome with ERCC5 mutation shows UV-independent increased susceptibility to apoptosis

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Abstract

Primary microcephaly, defined as head circumference below -3 SD at birth, is caused by genetic mutations leading to decreased mitotic rate and/or accelerated apoptosis of neural progenitors. A rare cause of severe microcephaly is cerebro-oculo-facial-skeletal syndrome (COFS), characterized by a catastrophic course and early death. The question was raised whether insufficient proliferation of neural progenitors, as proposed for other causes of primary microcephaly with prolonged survival, would be sufficient to explain the microcephaly and severe course. Mutations in DNA excision repair genes *ERCC1, -2 (XPD), -5 (XPG), -6 (CSB)* can cause COFS syndrome. We hypothesized that abnormal accelerated apoptosis would correlate with the presentation of COFS syndrome, compared to other DNA repair defects. We describe 7 new COFS individuals with *ERCC5* mutations who died in infancy. Fibroblasts of these patients are unable to repair UV-induced DNA damage. We have studied tendency to apoptosis in fibroblasts derived from individuals with COFS syndrome and microcephaly in order to test the hypothesis that tendency to apoptosis correlates with a congenital microcephaly. We observed in the COFS syndrome cells an increased susceptibility to cell death under stress conditions in the absence of UV radiation as a trigger. Apoptotic levels correlated with the severity of microcephaly and disease course. Additionally, *Ercc5* knockout mice showed increased apoptosis at 4 weeks in tissues including cerebral frontal cortex. We propose that primary microcephaly and lack of brain development in COFS syndrome, as example of severe DNA repair defect, is caused by abnormal apoptosis of neural progenitors, in part independent of externally (UV) induced DNA damage.

Introduction

Primary microcephaly, defined as head circumference at birth at or below -3 SD of expected, is a developmental disorder often caused by genetic mutations, but different hypotheses have been proposed about the mechanism of onset. Primary microcephaly is associated with variable course, e.g. mild to moderate intellectual disability (ID), epilepsy and only mild growth delay[1], or with severe neurological phenotype, progressive course and early death[2]. As a general mechanism, decreased proliferation of neural progenitors has been proposed, since many mutations involve genes responsible for regulation of mitosis[3,4]. Accelerated death of neuronal precursors leading to unscheduled apoptosis during brain development has also been proposed but there are very few examples providing evidence for this mechanism in animal models[5,6,7]. Another group of disorders variably associated with microcephaly are DNA repair disorders, a heterogeneous group of diseases caused by failure of one of the several mechanisms repairing DNA damage from endogenous metabolism (e.g reactive oxygen species) or from external causes such as irradiation, reactive chemicals etc. Some repair processes operate genome wide e.g global genome nucleotide repair (GG-NER) a multi-protein cut-and-patch repair system for a wide range of helix-distorting lesions, including the main UV-induced DNA lesions as well as numerous chemical adducts. Other repair pathways are associated with replication and/or transcription, e.g. transcription-coupled nucleotide excision repair (TC-NER), which specifically eliminates bulky adducts which block ongoing transcription to allow rapid resumption of the vital process of gene expression[8]. DNA repair disorders predispose to cancer and immune disease, primary growth failure, neurodegeneration and premature aging, all generally considered consequences of loss of cell proliferation control[8,9].

A rare, early onset DNA repair defect, cerebro-ocular-facial-skeletal (COFS) syndrome, presents with severe microcephaly at birth, cataracts and/or microphthalmia, facial dysmorphism, and arthrogryposis[10]. Affected individuals are usually small for gestational age and show failure to thrive, major feeding difficulties, sun sensitivity, failure to achieve developmental milestones and they frequently die in early infancy because of brain stem failure. Brain MRI typically shows simplified gyration of the cerebral cortex, enlarged lateral ventricles, cerebellar atrophy and delayed myelination[11]. Because of the presentation, COFS syndrome is considered to be a severe form of Cockayne syndrome, which has similar but milder abnormalities

and progresses more slowly. Based on complementation and sequence analysis, COFS has been associated with mutations in the same genes causing Xeroderma pigmentosum (XP) or Cockayne syndrome (CS)[10] (*ERCC1* (excision repair cross-complementing group 1; MIM 610758), *ERCC2/XPD* (MIM 610756), *ERCC5/XPG* (MIM 278780), *ERCC6/CSB* (MIM 214150)). Mutations in *ERCC5* also cause XP, the seventh complementation group G (XPG)[12]. Approximately 30% of XPG individuals have a neurological phenotype[13] however there is no adequate explanation for the difference in phenotype between CS and XP individuals. Several theories have been proposed including an effect of the type of mutation, DNA repair pathway involved and a possible role of premature death of neurons in the central nervous system[14,15,16].

Because of the catastrophic neurological phenotype associated with COFS we wondered whether the mechanism would be only related to insufficient proliferation or also to abnormal up regulation of cell death. For this purpose, we compared genetic mutations and susceptibility to apoptosis of living cells derived from seven new un-described COFS individuals with *ERCC5* mutations, one previously reported individual with *ERCC5* mutation and a mild neurological phenotype (XP) without microcephaly, and one “classic” CS individual with prolonged survival. Additionally we studied the occurrence of apoptosis in brains of *Ercc5* knockout mice, which show a severe phenotype comparable with COFS syndrome.

Clinical Reports

The majority of the COFS individuals presented in this report have not been described previously. We summarize here the clinical presentation of these individuals.

Case 1 – Individual FL 10E425.

The proband is the 2nd child of non-consanguineous Dutch parents. She was noted to have poor foetal growth at gestational week (GW) 26; amniocentesis showed normal female karyotype and fetal brain scans noted large ventricles and a small cerebellum. Examination at birth (37+4 weeks) showed a birth weight of 2105g, occipito-frontal circumference (OFC) of 30.4cm (-3SD), mild to moderate contractures of the major joints, facial dysmorphism with a sloping forehead, deep set eyes, micrognathia, broad nasal bridge and short palpebral fissures. Ophthalmological examination revealed no cataracts but pale optic discs, brainstem auditory evoked

response (BAER) revealed maximal perceptive deafness. She made no neurological progress and was naso-gastric (NG) tube-fed. A brain MRI showed enlarged ventricles, simplified gyral pattern of the cortex, hypoplastic corpus callosum, cerebellar vermis, cerebellar hemispheres, pons, optic nerves and lack of myelination (Figure 1A+B). She showed irregular respiration and died suddenly at 2 months of age.

Case 2 – Individual Y 94E0106. Clinical data of this individual have already been published by Hamel et al [17].

Case 3 – Individual L 96E116. The male proband is the first child of consanguineous Moroccan parents, born at 38 weeks gestation by caesarean section. During pregnancy growth retardation and oligohydramnios were noticed. Examination at birth showed microcephaly (OFC 30.1 cm -3SD), birth weight 1750 g, micrognathia with a high palate and flexion contractures of knees, ankles, wrists and elbows. Feeding difficulties were present from birth on; he was NG tube-fed. Due to feeding problems and frequent respiratory tract infections he was hospitalized from birth until age 3 months. He had seizures from age 4 months. Ophthalmological examination showed severe cataract in both eyes. A CT scan of brain revealed agenesis of the corpus callosum. He died at age 8 months.

Case 4 – Individual AA 01E0092 First pregnancy of consanguineous Moroccan parents. Examination at birth showed microcephaly (-3SD) with dysmorphic features; small deep set eyes, contractures of major joints and flexure contractures of fingers, and bilateral cataracts. He was NG tube- fed and at 1 month developed epilepsy. EEG showed focal right temporal irritability with secondary generalisation. By 5 months of age, OFC was -8 SD, he had made no neurological progress, had had several episodes of pneumonia and required home oxygen. MRI revealed a small cerebellum, large retro-cerebellar space, supratentorial reduced gyration, and subtle calcification in the thalami. He died at 8 months of age of pneumonia.

Case 5 – Individual H.A 02E0682 Brother of case 4, 2nd child born at 42/40 GW; at birth HC was 31.5cm (-3SD) with dysmorphic typical facial features, areflexia and joint contractures. He died at 26 days of pneumonia.

Case 6 – Individual S 07E0874. Female infant, at the 20 week routine ultrasound, showed microcephaly (-2.5SD). Examination shortly after birth showed bilateral microphthalmia, cataracts, short neck, small

thorax, contractures of thumbs, elbows, knees and hips and vertical talus. Dysmorphic features were short, sloping forehead, prominent nose, and long filtrum. Hypertonia of the limbs with hypotonia of the thorax and neck. She developed progressive failure to thrive, swallowing difficulties requiring tube feeding, bilateral perceptive deafness and epileptic spasms with hypsarrythmia on EEG. Brain MRI at 4 months of age showed hypoplasia of the cerebellar hemispheres and brain stem, normal pons, simplified gyration of the cerebral cortex, dilated lateral ventricles, decreased myelination with thin corpus callosum (figure 1C+D). She died of respiratory insufficiency at 17 months.

Case 7 – Individual M 00E0656. Examination after birth showed a female infant with OFC of 29.5cm (-2.5SD), hypertonia, small thorax, contractures of wrists and ankles, microphthalmia, cataract, neck and axial hypotonia. She had vesicoureteral reflux. By 11 months of age she exhibited progressive feeding and respiratory problems and epileptic spasms with hypsarrythmia on an EEG. Brain MRI at 5 months showed diffuse cerebral atrophy and lack of myelination of the white matter, thin corpus callosum, and progressive dilatation of the lateral ventricles. She died at approximately one year of pneumonia.

Case 8 – Individual D, **96E1041.** 2nd child of non-consanguineous parents (previous healthy older brother). At birth the female infant was noted to be dysmature, microcephalic (-3SD) with contractures of major joints and congenital cataracts. At 2 years of age she could roll from back to front and most of nutritional intake via a gastrosomy. On examination she was microcephalic (-8SD), with central hypotonia, lack of spontaneous movements, progressive contractures of hips and knees and fingers, she did not respond to light and required hearing aids. She had repeated admissions due to feeding difficulties, gastro-oesophageal reflux and aspiration pneumonias. She developed infantile spasms and absence seizures.EEG showed sporadic epileptic activity with an abnormal background. EMG showed polyneuropathy MRI showed generalised widened ventricular system, thin corpus callosum, hypoplasia of inferior vermis and diffuse hypomyelination. Clinical information indicates survival at least until the age of 4 years.

Case 9 – Individual H, 80E163. This individual with an *ERCC5* mutation had short stature, bird like face, sensorineural hearing loss, laryngeal dystonia and peripheral neuropathy with a mild clinical course. The clinical presentation has been described as the XP-G individual (case 15) by Anttinen et al in 2008[12].

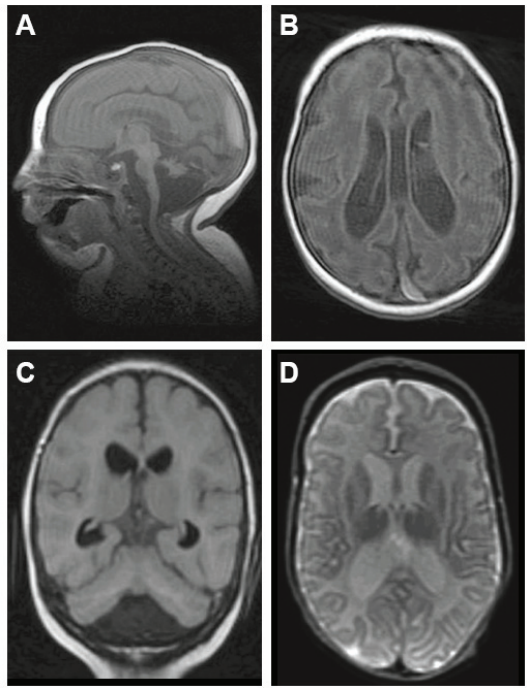

Figure 1. MRI of individuals 1 and 6A and B: MRI head of individual 1 performed at approximately 1 month of age. 1A: Sagittal T1 weighted view shows simplified gyral pattern of the cortex, normal corpus callosum, hypoplasia of the cerebellar vermis, hemispheres and pons. 1B: T1 weighted transversal view showing enlarged lateral ventricles, cavum septum pellucidum and simplification of the gyri.C and D: MRI head of individual 6 at approximately 4 months of age. 1C: T1 coronal weighted view showing hypoplasia of the inferior vermis and simplified gyri. 1D: T2 weighted transversal view showing enlarged lateral ventricles and simplified gyral pattern.

Materials and Methods

DNA repair studies and genotyping

GG-NER) and TC-NER were assayed in cultured skin fibroblasts from affected individuals and controls according to Jaspers *et al*.[18]. In these tests, XP and CS individuals show the same abnormalities as COFS[18]. After a specific NER defect was identified, cells were complemented with fibroblasts of individuals with known mutations and classified according to their complementation group[19]. Subsequently the genes were Sanger-sequenced according to their complementation group, with the exception of case 8.

Apoptosis Studies

Using cultured skin fibroblasts from case 1-7 (COFS), one individual with *ERCC6* mutation and a neurological phenotype (Case 8), one individual with *ERCC5* mutation and a mild non-neurological phenotype (Case 9) and as a positive control, fibroblasts from an individual with a *PNKP* mutation[7] and fibroblasts from an individual with confirmed *IER3IP1* mutation[5] and 5 healthy control cell strains, we tested for susceptibility to apoptosis. Apoptosis was detected in cells by staining with the FlicaTM (Fluorescent-Labelled Inhibitor of Caspases) apoptosis multi-caspase detection kit (Immunochemistry Technologies, LLC), which makes use of an inhibitor sequence of active caspases and was performed according to the manufacturer instructions. Percentage of apoptotic cells were calculated by scoring fluorescent cells before and after stress induction by 24 hours serum deprivation and then a further 24 hours exposure to 5 mM dithiothreitol (DTT) by two blinded investigators. Necrosis versus apoptosis was tested by vital staining exclusion with propidium iodide. The experiment was repeated three times and each time performed in triplicate.

Mouse Studies

The generation and characterization of a new, *bona fide Ercc5 (Xpg)* knockout mice, which accurately recapitulate the DNA repair defects of the human disease and closely mimic many clinical symptoms of the severe form of *ERCC5* defects in patients will be described in a separate publication (Barnhoorn *et al.*, submitted). All animals were of the same F1 C57BL6J/FVB hybrid background and had *ad libitum* access to standard mouse food (CRM pellets, SDS BP Nutrition Ltd; gross energy content 18.36 kJ/g dry mass, digestible energy 13.4 kJ/g) and water. The experiments were performed in accordance with the Principles of Laboratory Animal Care (NIH publication no. 86-23) and with the guidelines approved by the Erasmus University Animal Care Committee.

Histological procedures

Mice were anesthetized with pentobarbital and perfused transcardially with 4% paraformaldehyde. The brain was carefully dissected out and fixed overnight in 4% paraformaldehyde. Routinely, brain tissue was embedded in 10% gelatine blocks, rapidly frozen, and sectioned at 40 µm with a freezing microtome or stored at -80°C until use. Sections were processed, free-floating, using a standard avidin– biotin–immunoperoxidase complex method (ABC, Vector Laboratories, Burlingame, CA) with diaminobenzidine (0.05%; Sigma) as the chromogen.

Alternatively, brain specimens were fixed overnight in 10% buffered formalin, paraffin-embedded, sectioned at 5 µm, and mounted on Superfrost Plus slides. For immunohistochemistry, paraffin sections were deparaffinized, rehydrated in decreasing concentrations of ethanol, treated with 10 mM sodium citrate buffer, pH 6, in the microwave for antigen retrieval, and further processed using the ABC method.

A series of paraffin sections were employed for TdT-mediated dUTP Nick-End Labeling (TUNEL) assay using a commercial kit (Apoptag Plus Peroxidase in situ apoptosis detection kit, Millipore). Sections were deparaffinized and incubated as described by the manufacturer.

Primary antibodies (supplier; dilutions) used in this study were rabbit anti-p53 (Leica; 1:1000) and rat anti-Ki67 (Dako; 1:200). Biotinylated secondary antibodies from Vector Laboratories were used in a 1:200 dilution. Levels of p53 were assessed using gelatin sections, whereas TUNEL and Ki-67 was determined on paraffin sections using 4 animals per group. Immunoperoxidase-stained sections were analysed and photographed using an Olympus BX40 microscope and a ColorView digital camera.

Results

DNA repair

Fibroblasts from Cases 1-9 were tested for various nucleotide excision repair (NER) endpoints using UV as a source of DNA damage including sensitivity to UV light. Both GG-NER and TC-NER were grossly abnormal in COFS individuals (1 -7). Cells from the CS individual 8 show as expected a strongly reduced (albeit not completely absent) TC-NER activity and normal GG-NER in the wild type range, i.e. normal transcription-independent DNA repair. In individual 9 (XP) only GG-NER was tested and was deficient (Table 1).

Sequencing/Complementation Analysis

The *ERCC5* mutations in individuals 1-7 and other affected individuals are presented in table 2. The results indicate that all the COFS individuals had a biallelic frame-shift mutation in *ERCC5*, among these the p.925fs55X being a well-known Moroccan founder mutation[17]. Case 1 showed a deletion in exon 11 at position 811 (p.Ser811f521X) and a second mutation was found in cDNA on qRNA – deletion of 16 nucleotides in the terminal part of exon 9 resulting in a frameshift from Gln^{729} (p.Gln729fs11X).

The missense *ERCC5* mutation (case 9) with milder XP phenotype has been reported earlier[12]. The cells bearing mutations in *IER3IP1* and *PNKP* have been previously reported[5,6].

Apoptosis experiments in individual fibroblasts

We tested *in vitro* susceptibility to apoptosis on cells from individuals 1-9. As a positive control we used cells from an individual bearing *PNKP* mutation[6] and cells bearing an *IER3IP1* mutation[5], which had previously been shown to have an increase tendency to apoptosis. We found that cells from individuals with *ERCC5* mutations, microcephaly and severe neurology had an increased tendency to undergo apoptosis (figure 2A). In case 8 who has severe neurology and *ERCC6* mutation, and case 9 (*ERCC5* mutation and normal head circumference) apoptosis levels were similar to control fibroblasts. Notwithstanding the limited number of individual cell lines, this data suggests a clear correlation between the tendency of the cells to undergo apoptosis *in vitro*, the type of mutation, the residual DNA repair activity and the severity of the clinical presentation. Without DTT treatment the levels of apoptosis were low and there was no significant difference between all cells lines. Considering the extreme susceptibility of *ERCC5* fibroblasts to X-radiation and UV we could not test apoptosis after irradiation. Similarly mouse embryonic fibroblasts (MEFs) from knockout mice did not survive long enough to be tested for apoptosis (data not shown).

Ercc5 KO mouse studies

The data from the human cells is suggestive, but does not demonstrate the role of abnormal apoptosis during brain development. Since data from human brain is lacking, we decided to investigate the developing brain of a mouse model displaying striking similarity to the human *ERCC5* phenotype, including progressive neurological abnormalities reminiscent of severe *ERCC5* patients (Barnhoorn *et al.*, submitted).

Ercc5 (Xpg) knockout mice have a tendency to slightly lower brain weights at postnatal day one, mimicking COFS (unpublished data). We tested levels of spontaneous apoptosis in these animals and observed a significant increase in apoptotic cells in the frontal cortex at the age of 4 week as shown by positive staining for TUNEL and p53 (figure 2B and 2C). This was also observed in other regions of the central nervous system, including cerebellum and retina (Barnhoorn et al., submitted). No difference in Ki-67 staining was observed (figure 2D), suggesting normal cell proliferation levels between *Ercc5* knockout mice and littermate controls.

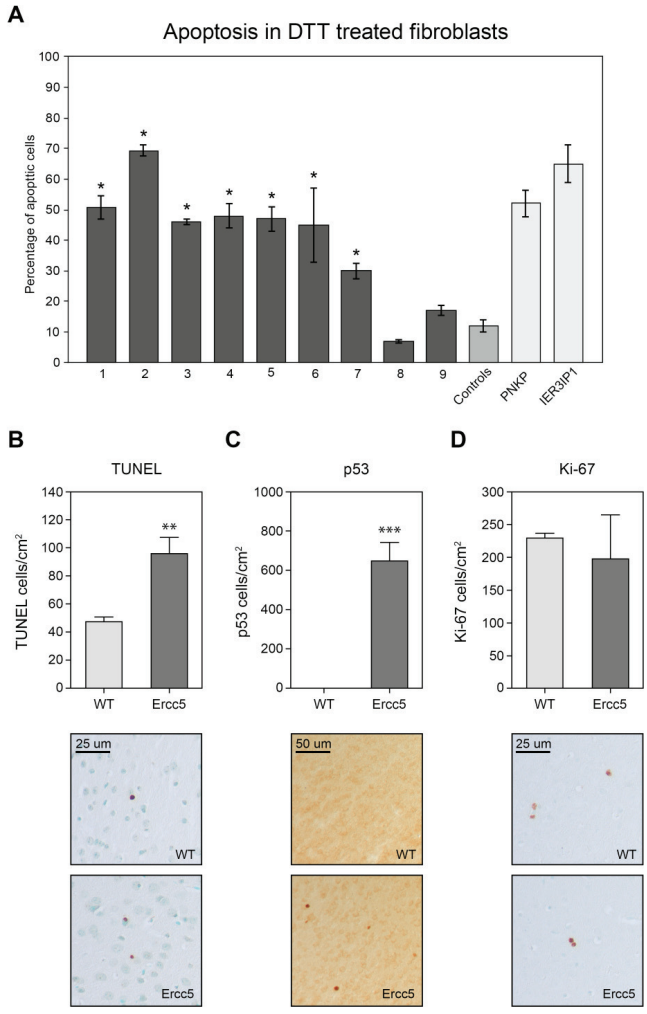

Figure 2. Test for apoptosis in fibroblasts and mouse brain Figure 2: A: apoptosis in DTT treated fibroblasts. Individuals 1-7 who exhibit signs of COFS syndrome and have a homozygous XPG mutation (see Table 2). Fibroblasts have an increased tendency to apoptosis. Fibroblasts from individual 8 with Cockayne syndrome (CSB complementation group) had a lower level of apoptosis, in the control range. Fibroblasts from individual 9 with XPG mutation and no microcephaly exhibited levels of apoptosis just above the control range. All apoptosis is tested after treatment with 5mM DTT in comparison to 5 control cell lines and two positive control fibroblasts (individuals with a *PNKP* and *IER3IP1* mutations respectively) on three separate occasions. Rates are shown as a mean of three experiments (+/- SEM); significant differences (*, $p<0.05$) were calculated by the Student T test. Without DTT provocation apoptosis levels were between 6-10% for patients and controls.
B: Quantification of TUNEL-positive cells per cm^2 in frontal cortex sections of 4 weeks old XPG-/- and WT mice. Error bars indicate SEM; significant differences (**, $p<0.01$) were calculated by the Student T-test; n=4 animals/group.

Discussion

DNA repair disorders are clinically heterogeneous and neurological features are common. A diverse range of DNA repair defect disorders can be associated with microcephaly i.e. COFS syndrome, Nijmegan Breakage Syndrome (NBS, MIM 251260), Fanconi Anemia (FA, MIM 227650), Bloom Sydrome (BS, MIM 210900), Ligase 4 syndrome (*LIG4* syndrome MIM 606593), CS and more recently Microcephaly, seizures and developmental delay (MCSZ syndrome caused by mutations in *PNKP,* MIM 613402)[7]. Some DNA repair disorders mostly exhibit a primarily neurodevelopmental phenotype (NBS, COFS), others a neurodegenerative phenotype, e.g. CS, spinocerebellar ataxia-neuropathy type 1 (SCAN1, *TDP1* mutation, MIM 607250). Microcephaly is common in disorders with a developmental phenotype.

However, some DNA repair disorders, such as MCSZ, present with both a congenital microcephaly and a degenerative phenotype[6]. The pathogenesis of congenital microcephaly is commonly ascribed to insufficient neuroprogenitors proliferation, because of mitotic dysregulation by the centrosome[20,21,22]. Microcephaly caused by DNA repair disorders might represent an exception to this general rule. COFS syndrome presents with congenital microcephaly and severe neurological course leading to early death. As COFS can be a manifestation of severe mutations in XP/CS genes, such as *ERCC1*, *ERCC2*, *ERCC5* and *ERCC6*, the same mechanisms playing a role in the pathogenesis of neurological symptoms in Xeroderma Pigmentosum and Cockayne Syndrome could be causative. In 1978, XP fibroblasts strains showed sensitivity to UV radiation by losing ability of colony formation after irradiation, the sensitivity correlating with the degree of neurological symptoms and type of presumed genetic mutation[15]. These and other authors assumed the mechanism to be a lack of protection against premature neuronal death as an explanation of neurodegeneration[12,15]. The accumulation of unrepaired DNA oxidative lesions in brain has been claimed to be the mechanism leading to progressive neuronal death and neurodegeneration in XP individuals[23]. Both arrest of normal cell cycle i.e. proliferation and activation of cell death cascade could be responsible for the developmental defects, such as microcephaly in COFS syndrome. In *MCPH1* mutation, one of the autosomal recessive primary microcephalies, such a dual mechanism probably underlies the pathogenesis of microcephaly[21,24,25,26]. We observed an abnormal susceptibility to apoptosis of cells derived form individuals with COFS syndrome caused by *ERCC5* mutations. In COFS syndrome

a defective proliferation is not supported by the data we have from the *Ercc5* mouse model, which shows no difference in proliferation between the knockout mouse and the wild type, suggesting the predominant mechanism is increased apoptosis.

There are several links, which could explain the onset of microcephaly as consequence of abnormal apoptosis, although the mechanism leading to premature cell death might be different in each DNA repair disorder. *PNKP* mutations determine early apoptosis of neural progenitors[7]. The PNKP enzyme has a dual function as a polynucleotide 3' phosphatase and a polynucleotide 5' hydroxykinase, remodelling both the 5' and 3' end of damaged DNA and binding to XRCC1, one of the effectors of BER[27]. PNKP is activated and recruited by ATM and ATR. PNKP is also involved in the repair of double-strand breaks (DSB) related to non-homologous end joining (NHEJ) occurring during mitosis. DSB repair is particularly important in dividing cells during development. In this pathway PNKP protein directly interacts (via the third forkhead domain) with the scaffold XRCC4 and activates LIG4[27,28]. Human mutations in *LIG4* and *ATR* (Microcephalic primordial dwarfism type 1, MPD 1, Seckel syndrome type 1, MIM 210600) are causative of syndromes with primary microcephaly. *Lig4* knockout mice have also shown abnormal apoptosis in the brain[29].

The observation of DNA repair defects in primary microcephaly syndromes such as Seckel syndrome type 1, MCSZ and correlation to abnormal apoptosis as the pathogenic mechanism, triggered us to test cells from a larger series of individuals with COFS syndrome and corresponding valid animal models. Most of the available cell strains were from individuals belonging to the *XPG/ERCC5* group. ERCC5 protein is important for DNA repair and is responsible for the 3`incision in Nucleotide Excision Repair (NER) via both the global genome repair (GGR) and transcription coupled repair (TCR) pathways[30]. ERCC5 directly interacts with the other XP proteins such as ERCC1 and ERCC4, which are also associated with COFS syndrome. The NER pathway is an important excision pathway for removing mutations derived from a broad range of insults. ERCC5 (XPG) alone with ERCC6 (CSB) may also have a role in base excision repair processes which is used to repair oxidative damage which could help explain why developing neurons are preferentially affected[31]. Additionally, ERCC5 has been suggested to be a negative regulator of apoptosis and *ERCC5* mutant cell strains are more susceptible to apoptosis upon UV irradiation than other DNA repair defective cells[32]. However, apoptotic response of *ERCC5* mutant cells did not seem to depend on the extent of unrepaired DNA damage[33]. Here we show increased susceptibility to apoptosis *in vitro* in cells from

ERCC5 individuals, through activation of multiple caspases (among which caspase 3 and 9), upon low doses of the cysteine reducing agent DTT, but without the UV trigger, suggesting an intrinsic vulnerability of these cells. We also observe a correlation between this susceptibility and the most severe neurological phenotype, including congenital microcephaly and early death, although the numbers of cell lines tested is limited. This is similar to earlier observation of sensitivity to UV radiation and capacity of colony formation[15]. Additionally, in strong support of this interpretation elevated apoptosis levels were observed *ex vivo* by both TUNEL and p53 staining in developing brains of mouse models for COFS in the *Ercc5* knockout mice, whereas cell proliferation remains unaffected in these animals.

We therefore hypothesize that in COFS syndrome the mechanism leading to neuronal death and insufficient brain growth, i.e. microcephaly, as well as insufficient maintenance of neural lineages after birth, i.e. neurodegeneration, might be abnormal up regulation of apoptosis. Accelerated neurodegeneration is associated with DNA repair defects that present after a normal developmental phase and is thought to be due to increased cell loss due to a defective NER/TCR pathway. The exact pathway is unclear but *Ercc1* KO mice have been shown to exhibit slow and progressive neurodegeneration as well as increased cell loss in the cortex and hippocampus with increased p53 and caspase staining indicating an increase in apoptosis of the neurons[34]. It is therefore possible that upregulation of apoptosis in COFS syndrome contributes to both developmental abnormalities i.e. microcephaly and accelerated neurodegeneration leading to early demise.

It will be interesting in the future to study to which extent, in this and other DNA repair disorders, apoptosis plays a role in the onset of primary growth defects such as microcephaly and which apoptotic pathways are triggered by these mutations and which combination of factors determines the clinical variability and progression.

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Table 1. Biochemical DNA repair tests (global NER and transcription-coupled NER) in fibroblasts from seven COFS syndrome individuals, a Cockayne syndrome (CS), an XP individual and control cell strains.

Individual/ complementation group	Cell Line	Global NER Unscheduled DNA synthesis (Nuclear 3H-thymidine incorporation after 17h)	Transcription -coupled NER (DNA synthesis 17h after UV irradiation, 3J/m2
1 COFS/XPG	10E425	6%	8%
2 COFS/XPG	94E106	4%	19%
3 COFS/XPG	96E116	4%	13%
4 COFS/XPG	01E0092	14%	8%
5 COFS/XPG	02E0682	9%	5%
6 COFS/XPG	07E0874	9%	6%
7 COFS/XPG	00E0656	2%	9%
8 CS/CSB	96E1041	67%	24%
9 XP/XPG	80E0163	4%	Not determined
10 Control range	N=50	50-80%	100%

Table 2. Mutations in affected individuals at amino acid level.

Individual	Cell line	Diagnosis/phenotype	Gene	Mutation	Ref
1	10E425	COFS Microcephlay at birth (-3 SD) Died at 2 months	*ERCC5 (XPG)*	p.Ser811Valfs21X /p.Gln729Argfs11X	This paper
2	94E0106	COFS (XP/CS) Died at 7 months Microcephaly at birth (-3SD))	*ERCC5 (XPG)*	p.Pro925Profs55X	Hamel et al [17]
3	96E0116	COFS	*ERCC5 (XPG)*	p.Pro925Profs55X	This paper
4	01E0092	COFS Microcephaly at birth Epilepsy, died at 8 months	*ERCC5 (XPG)*	p.Pro925Profs55X	This paper
5	02E0682	COFS Microcephaly at birth, respiratory difficulties, died at 26 days	*ERCC5 (XPG)*	p.Pro925Profs55X	This paper
6	07E0874	COFS Microcephaly at birth (2.5SD) Epilepsy, died at 16 months	*ERCC5 (XPG)*	p.Pro925Profs55X	This paper
7	00E0656	COFS Microcephaly at birth (-2.5SD) Epilepsy, died at 11 months	*ERCC5 (XPG)*	p.Pro925Profs55X	This paper
8	96E1041	CS with neurological phenotype, microcephaly (-3SD), feeding difficulties, severe developmental delay	*ERCC6 (CSB)*	Result based on complementation analysis	This paper
9	80E0163	XP (short stature, deafness, peripheral neuropathy, mild course, no tumours)	*ERCC5 (XPG)*	p.Gly766X/ p.Ala1018Metfs1X	Anttinen A et al [12]

References

1. S Mahmood , W Ahmad , H Muhammad (2011) Autosomal recessive primary microcephaly (MCPH): clinical manifestations, genetic, heterogeneity and mutation continuum. Orphanet Journal of Rare Diseases, 6:39e.

2. A Rajab, C. M Manzini, G Mochida, C Walsh, E Ross (2007) A novel form of lethal microcephaly with simplified gyral pattern and brain stem hypoplasia, Am.J.Med.Gen.143A:2761-7.

3. J.A Barkovich, R Guerrini, R.I Kuzniecky, G.D Jackso, W.B Dobyns (2012) A developmental and genetic classification for malformations of cortical development: update 2012. Brain 135:1348-69.

4. GK Thornton, C.G Woods, Primary Microcephaly: do all roads lead to Rome? (2009) Trends in Genetics. 25:11:501-10.

5. C.J Poulton, R Schot, S Kheradmand Kia, M Jones, F W. Verheijen, H Venselaar, M.C.Y de Wit, E de Graaff, A.M Bertoli-Avella, G.M.S Mancini (2011) Microcephaly with simplified gyration, epilepsy, and infantile diabetes linked to inappropriate apoptosis of neural progenitors. Am J Hum Genet 89:265-276.

6. C.J Poulton, R Oegema, D Heijsman, J Hoogeboom, R Schot, H Stroink, M.A Willemsen, F.W Verheijen, P van de Spek, A Kremer, G.M Mancini (2013) Progressive cerebellar atrophy and polyneuropathy: expanding the spectrum of PNKP mutations, Neurogenetics. 14:43-51.

7. J Shen, E.C Gilmore, C.A Marshall, M Haddadin, J.J Reynolds, W Eyaid , B Barry, D Gleason, K Allen, V.S Ganesh, B.S Chang, A Grix, R.S Hill, M Topcu, K.W Caldecott, A.J Barkovich, C.A Walsh (2010) Mutations in PNKP cause micocephaly, seizures and defects in DNA repair. Nat Genet. 42:24 5-9.

8 J.H. Hoeijmakers DNA damage,aging and cancer (2009) New Engl J Med 361:1475-8510.

9. P.J McKinnon, K.W Caldecott (2007) DNA Strand Break Repair and Human Genetic Disease. AnnuRev Genom Hum Genet 8:37-55.

10. V Natale (2010) A Comprehensive Description of the Severity Groups in Cockayne Syndrome Am J Med Genet. 155A:1081-95.

11. M Koob, V Laugel, M Durand, H Fothergill, C Dalloz, F Sauvanaud, H Dollfus, I.J Namer, J.L Dietemann (2010) Neuroimaging in Cockayne Syndrome, Am J Neuroradiol 31:1623-30.

12. A Anttinen, L Koulu, E Nikoskelainen, R Portin, T Kurki, M Erkinjuntti, NG Jaspers, A Raams, M.H Green, A.R Lehmann, J.F Wing, C.F Arlett, R.J Martila (2008) Neurological symptoms and natural course of xeroderma pigmentosum. Brain 131:1979-89.

13. P Bradford, A.M.Goldstein, D Tamura, S.G Khan, T Ueda, J Boyle, K Oh, K Imoto, H Inui, S Moriwake, S Emmert, K.M Pike, A Raziuddin, T.M Piona, J.J DiGiovanna, M.A Tucker, K.H Kraemer (2011) Cancer and neurologic degeneration in xeroderma pigmentosum: long term follow up characterises the role of DNA repair. J Med Genet 48:168-17019.

14. E Jaakola, A Mustonen, P Olsen, S Miettinen, T Savoja, N.G Jaspers, H Shao, B.L Wu, J Ignatius (2010) ERCC6 founder mutation identified in Finnish individuals with COFs syndrome. Clin Genet 78:541-547.

15. Andrews A.D, Barrett S.F, Robbins J.H (1978) Xeroderma pigmentosum neurological abnormalities correlate with colony-forming ability after ultraviolet radiation. Proc Nat Acad Sci USA 75**:**1984-1988.

16. Soltys D, Rocha C.R.R, Lerner L.K, de Souza T.A, Munford V, Cabral F, Nardo T, Stefanini M, Sarasin A, Cabral-Neto J.B, Menck C.F.M (2013) Novel XPG (ERCC5) mutations affect DNA repair and cell survival after ultraviolet but not oxidative stress. Hum Mutat 34:348-489.

17. Hamel B.C.J, Raams A, Schuitema-Dijkstra A.R, Simons P, van der Burgt I, Jaspers N.J, Kleijer W.J (1996) Xeroderma pigmentosum-Cockayne syndrome complex: a further case. J Med Genet 33:607-610.

18. Jaspers N.G, Raams A, Silengo M.C, Wijgers N, Niedernhofer L.J, Robinson A.R, Giglia-Mari G, Hoogstraten D, Kleijer W.J, Hoeijmakers J.H, Vermeulen W (2007) First reported individual with Human ERCC1 Deficiency has Cerebro-Oculo-Facial-Skeletal Syndrome with a Mild Defect in Nucleotide Excision Repair and Severe Developmental Failure. Am J Hum Genet 80:457-66.

19. Vermeulen W, Jaeken J, Jaspers N.G, Bootsma D, Hoeijmakers J.H (1993) Xeroderma pigmentosum complementation group G associated with Cockayne Syndrome, Am J Hum Genet 53:185-192.

20. Kaindl A.M, Passemard S, Kumar P, Kraemer N, Issa L, Zwirner A, et al (2010) Many roads lead to primary autosomal recessive microcephaly. Prog Neurobiol 2010 90: 363-83.

21. Gruber R, Zhou Z, Sukchev M, Joerss T, Frappart PO, Wang ZQ (2011) MCPH1 regulates the neuroprogenitor division mode by coupling the centrosomal cycle with mitotic entry through the Chk1-Cdc25 pathway. Nat Cell Biol 2011 13: 1325-34.

22. Nigg E.A, Raff J.W (2009) Centrioles,centrosomes, and cilia in health and disease. Cell 139:663-78.

23. Emmert S, Slor H, Busch D.B, Batko S, Albert R.B, Coleman D, Khan S.G, Abu-Libdeh B, DiGiovanna J.J, Cunningham B.B, Lee M.M, Crollick J, Ueda T, Hedayati M, Grossman L, Shahiavi T, Cleaver J.E, Kraemer K.H (2002) Relationship of neurologic degeneration to genotype in 3 xeroderma pigmentosum group G individuals. J Invest Dermatol 118 :972-982.

24. O`Driscoll M, Jeggo P.A (2008) The Role of the DNA damage response pathways in brain development and microcephaly: Insight from human disorders. DNA Repair 7 :1039-1050.

25. O' Driscoll M, Jackson A.P, Jeggo P.A (2006) Microcephalin: a causal link between impaired damage response signalling and microcephaly. Cell Cycle 5:20 :339-2344.

26. Yang S.Z, Lin F.T, Lin W.C (2008) MCPH1/BRIT1 cooperates with E2F1 in the activation of checkpoint DNA repair and apoptosis. EMBO rep. 9:907-15. et al.

27. K.W Caldecott (2008) Single strand break repair and genetic disease Nat Rev Genet 9 :619-631.

28. Weinfeld M , Mani R.S, Abdou I ,Aceytuno R.D ,Glover J.N (2011) Tidying up loose ends: the role of polynucleotide kinase/phosphatase in DNA strand break repair. Trends Biochem Sci 36:262-271.

29. Gilmore E.C, Nowakawski R.S, Caviness V.S, Herrup K (2000) Cell birth, Cell death, Cell diversity and DNA breaks: how do they all fit together. Trends Neurosci 23:100-105.

30. de Boer J, Hoeijmakers J.H (2000) Nucleotide excision repair and human syndromes. Carcinogenesis. 21:453-460.

31. Nouspikel T, Lalle P,Leadon S.A, Cooper P.K, Coooper S.G, Clarkson S.G (1997) A common mutational pattern in Cockayne syndrome patients from xeroderma pigmentosum group G: implications for a second XPG function. Proceedings of the National Academy of Sciences of the United States of America 94**:**3116-3121.

32. Clément V, Dunand-Sauthier I.Wiznerowicz M Clarkson S.G (2007) UV-induced apoptosis in XPG-deficient fibroblasts involves activation of CD95 and caspases but not p53. DNA Repair 6:602-614.

33. Clément V, Dunand-Sauthier I, Clarkson S.G (2006) Suppression of UV-induced apoptosis by the human DNA repair protein XPG. Cell Death Differ. 13:478-88.

34. Sepe S, Payan-Gomez C.E, Milanese C, Hoeijmakers J.H, Mastroberadino P.G (2013) Nucleotide excision repair in chronic neurodegenerative disease. Accepted for publication in DNA repair.

Chapter 4

Microcephaly and Cortical Malformations

Severe presentation of WDR62 mutation: is there a role for modifying genetic factors?

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Abstract

Mutations in *WDR62* are associated with primary microcephaly; however they have been reported with wide phenotypic variability. We report three individuals with novel *WDR62* mutations who illustrate this variability. One lacks neuromotor development and has severe pachygyria on MRI, another has only delayed speech and motor development and moderate polymicrogyria, and the third has an intermediate phenotype. We observed a rare copy number change of unknown significance, a 17q25qter duplication, in the severely affected individual. The 17q25 duplication included an interesting candidate gene, tubulin cofactor D (*TBCD*), crucial in microtubule assembly and disassembly. Sequencing of the non-duplicated allele showed a *TBCD* missense mutation, predicted to cause a deleterious p.Phe1121Val substitution. Sequencing of a cohort of five individuals with *WDR62* mutations, including one with an identical mutation and different phenotype, plus twelve individuals with clinical microlissencephaly and another individual with mild intellectual disability (ID) and a 17q25 duplication, did not reveal *TBCD* mutations. Although definitive evidence is lacking, it is possible that additional genetic factors contribute to modify and aggravate the *WDR62* phenotype; Additional observations are needed to confirm whether *TBCD* is one of these factors.

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Microcephaly is defined as a head circumference of more than 3 SD below the expected for age and sex. Primary microcephaly is a developmental defect that can present with a normal or simplified gyral pattern of the cerebral cortex (MSG), thickened cortex (microlissencephaly, MLIS) or disorganized cortex (e.g. polymicrogyria, MIC+PMG). Primary microcephaly with a normal or thin cortex, has been described as an autosomal recessive disorder (MCPH) based on mutations in at least eight genes (*MCPH1, WDR62, CDK5RAP2, ASPM, CENPJ, STIL, CEP135 and CEP152*)[1]. *ASPM* and *WDR62* account for approximately 50% of the diagnoses[2]. There is usually no clinical heterogeneity among patients with MCPH; a phenotype of near normal motor development with moderately compromised cognition, although epilepsy has been reported[3]. The genes mutated encode a diverse range of proteins but with the majority involved in centrosomal function which leads to decreased proliferation of neural progenitors[4]. Major exceptions to the phenotypic similarity are the disorders associated mutations in *WDR62*[5]. Next-generation sequencing has identified mutations in *WDR62* in patients who would not have previously been grouped together for linkage studies[5,6].

WDR62 contains fifteen WD-type repeat domains typical for cytoskeleton interacting proteins[7]. Although function is unknown, it is expressed transiently in the neocortex during the period of neurogenesis in a pattern identical to *ASPM*[6]. Some authors have shown WDR62 to be associated with spindle poles during mitosis and to have cell cycle-dependent localization[8,9,10], implying a role in centrosomal function. Unlike the mutations in other MCPH genes, missense and null mutations in *WDR62* are distributed throughout the gene[6,8,10,11]. Additionally, *WDR62* mutations are associated with disorders of neuronal migration (lissencephaly) and cortical organization (polymicrogyria), and the degree of intellectual disability in patients is extremely variable (table 1)[6,8,10,11]. The difference in phenotype has been proposed by Nicholas *et al*. to be due to the type of mutation; with nonsense mutations leading to a more severe phenotype [8]. However, this correlation is not borne out since many of the nonsense mutations described cause mild phenotypes and some patients with missense mutations have very severe phenotypes (table 1)[5,6,10].

Murdock *et al*. proposed that the difference could be explained by environmental factors (e.g maternal diabetes) or by additional mutations

identified by whole exome sequencing (*GLI2* and *KIAA1598*) that provide an additive effect in the patients with abnormal cortical architecture and a more severe phenotype[11]. The use of comparative genome hybridization (CGH) has generated much data about copy number variation (CNVs), which harbour genes causative of syndromic and non-syndromic intellectual disability (ID)[12]. Similarly a large number of rare CNVs of unknown significance have been detected in ID individuals, classified as variants of unknown significance (VUS). In the clinical setting, finding a VUS does not usually explain the phenotype, however it can be contributory[12]. Additionally, it is possible that an uncommon variation unmasks a recessive disorder with a pathogenic mutation on the other allele[13].

We observed three individuals with primary microcephaly and novel *WDR62* mutations, greatly differing in their clinical presentation, one with an atypically severe phenotype (individual 1). We tested the hypothesis that additional factors contribute to the phenotypic variability. The detailed clinical presentation is described in the supplemental data.

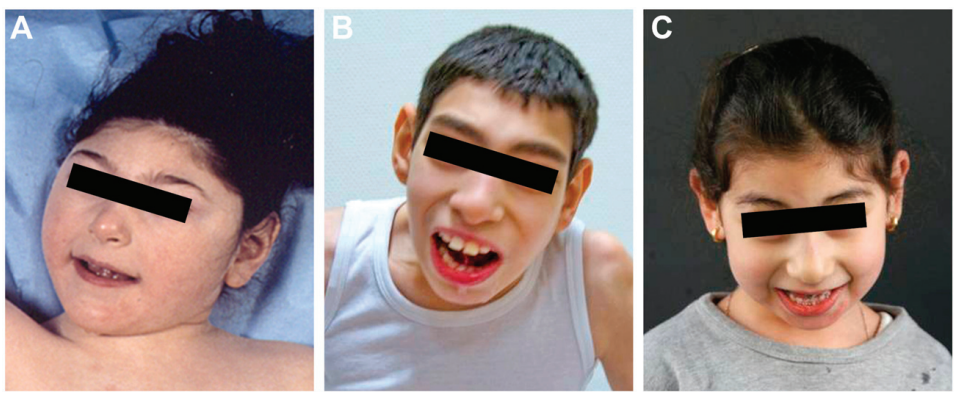


Figure 1. Clinical Photographs A: Individual 1 at 5 years of age showing extreme microcephaly, as well as very small forehead and skull and upslanted palpebral fissures. B: Individual 2 at 13 years of age, showing microcephaly, prominent ears, drooling. C: Individual 3 at 6 years of age. Facial features included a sloping forehead, small chin, prominent nose, upward slant of the eyelid, prominent lower lip.

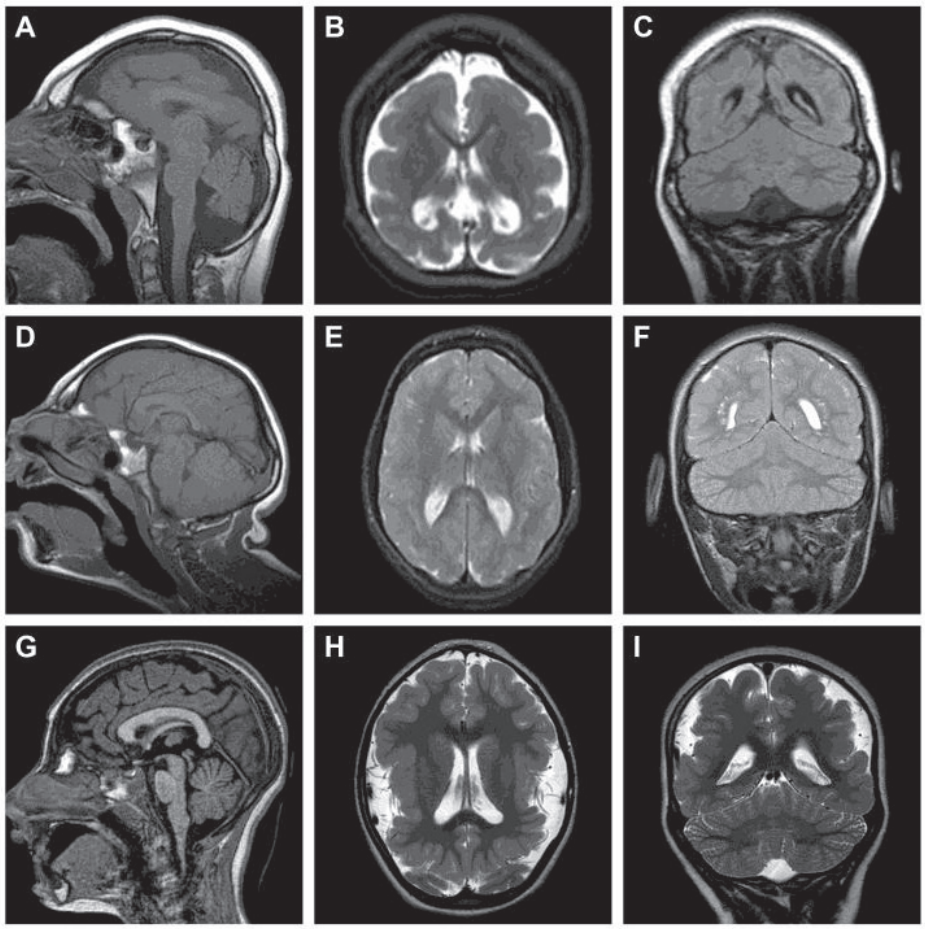


Figure 2. Brain MRIs A-C Brain MRI Individual 1 performed at 5 years of age A: Sagittal T1 weighted view showing sloping forehead and flattened occiput, grossly simplified gyral pattern and normal cerebellum. Dysplasia of the corpus callosum is partially visible. Please note the extreme discrepancy between cerebral and cerebellar size compared to individual 2 and 3. B: T2 weighted transverse view, showing simplified gyration and moderately thickened cortex. C: Coronal FLAIR view showing occipital lobes smaller than cerebellar hemispheres. D-F Brain MRI of individual 2. D: Sagittal T1 weighted view showing sloping forehead, moderate simplified gyral pattern, dysplastic corpus callosum and normal cerebellum. E: T2 weighted transverse view showing a moderately simplified gyral pattern and moderately thickened cortex. F: T2 weighted coronal image again showing simplified gyration and normal cerebellum. G-H: Brain MRI of individual 3. G: T1 weighted sagittal view showing microcephaly, normal corpus callosum and cerebellum; H:T2 weighted transverse view showing symmetrical polymicrogyria of temporal, parietal and frontal lobe with increased intra- and extracerebral space. I: T2 weighted coronal view showing polymicrogyric cortex and normal cerebellum. Remarkable in all three patients is the small anterior ventricle with a slightly enlarged posterior part of the ventricles.

Results

The results of Sanger sequencing of *WDR62* exons and intron-exon boundaries (Ensembl WDR62_201) in all three patients has been summarized in Table 1, together with all the mutations and phenotypes described in the literature. The mutations in three additional, previously undescribed patients are included. Patients 1 and 6 are homozygous for the mutation c.1605_1606insT resulting in a premature stop codon (p.Glu536*). Patients 2, 4 and 5 all have frame-shift mutations resulting in premature stop mutations. Patient 3 is homozygous for a missense mutation leading to a p.Arg863His substitution. Both parents of patient 3 were heterozygous for the change, which was predicted to be “probably deleterious” by the Polyphen2 and SNAP algorithms. This change is not reported in dbSNP databases nor in healthy individuals of the 1000 Genome Project. *IER3IP1* was sequenced in patient 3 because of the combination of microcephaly and childhood diabetes [14] and no mutation was found.

On routine Affymetrix GeneChip Human Mapping 250K SNP array performed on individual 1, copy number analysis indicated a duplication on chromosome 17q25qter from base pair position 80559167-tel (hg 19; last SNP before duplication rs3794716) (figure 3) which contains 13 genes (Supplemental Table S1 and figure 3). The duplication was confirmed by genomic quantitative PCR (qPCR) as being present in the proband and the asymptomatic mother. An overlapping duplication is described in the Toronto database of genomic variants (DGV) in one healthy individual. Within the duplicated area, tubulin cofactor D (*TBCD*) attracted our attention as the only gene possibly contributing to the phenotype (Supplementary table S1). TBCD is involved in microtubule assembly and disassembly and is essential for spindle microtubule dynamics[15,16] which are crucial during cortical development. Mutations in tubulin subunit genes and genes encoding microtubule-associated proteins such as *α-Tubulin 1A, LIS1* and *DCX* lead to lissencephaly or pachygyria due to aberrant neuronal migration[16,17,18]. Mutations in tubulin cofactors have been predicted to cause cortical malformations in humans[19]. As the MRI phenotype of individual 1 was rather reminiscent of pachygyria we decided to analyze *TBCD* in depth. On sequencing *TBCD* (Ensembl transcript TBCD_001) in the proband and the parents, a novel missense mutation in exon 35 was identified, c.3361T>G, which results in an amino acid change from phenylalanine to valine (p.Phe1121Val) (figure 3). The change was absent in the mother, the father was heterozygous and it was present in the patient with an estimated ratio of 1/3 alleles, compatible with the presence of a duplicated wild-type allele (figure 3).

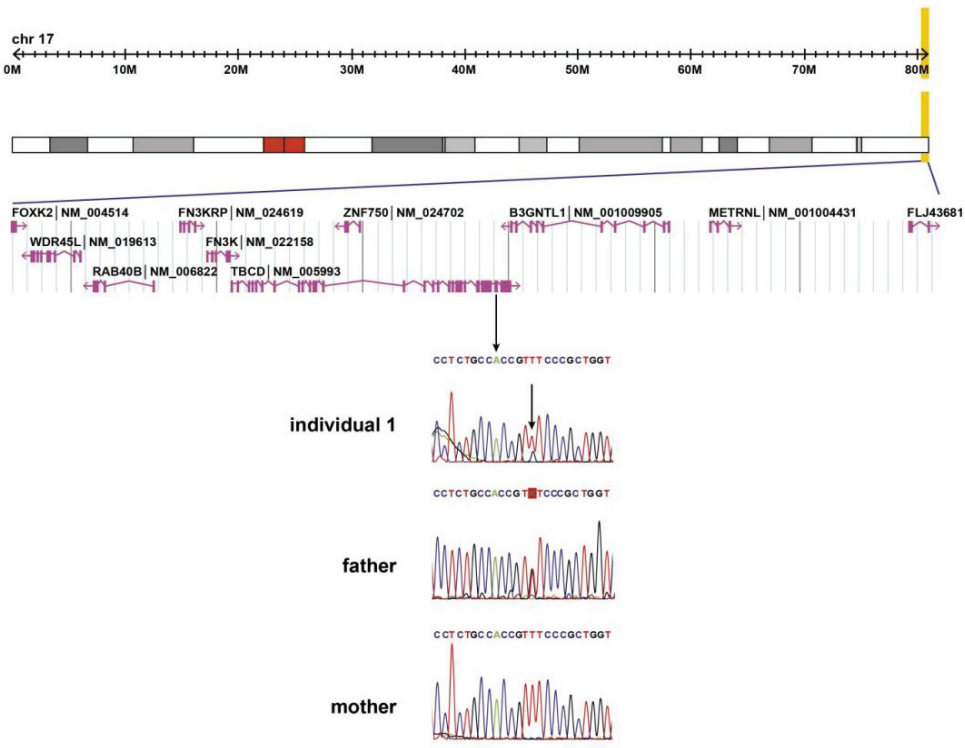


Figure 3. TBCD locus analysis Patient 1: CNV (proband and mother) on chromosome 17 (yellow bar) with the genes within the duplication region. Underneath, Sanger sequencing showing a novel missense mutation in TBCD, c.3361T>G, in individual 1 at a ratio1/3, father who is heterozygote for the change and the mother (wild type).

The p.Phe1121Val is predicted to be pathogenic by Polyphen-2 and SNAP (supplemental data). This mutation was not found in 206 chromosomes from ethnically matched individuals and is not reported in dbSNP130, nor in the 1000 Genome Project database, indicating a very low allele frequency. No difference in the levels of *TBCD* mRNA expression was detected by RT-qPCR between patient and control skin fibroblasts cultured under standard serum conditions (data not shown). We sequenced *TBCD* in individuals 2-6, including individual 6 who has the same *WDR62* mutation (table 1) and a moderate phenotype: no mutation was found. To test whether biallelic *TBCD* mutations themselves are a frequent cause of cortical malformation, we sequenced twelve patients with micro-lissencephaly (Aix Marseille University, France) and no mutations in *TBCD* were found. We sequenced *TBCD* in an individual with mild intellectual disability and autistic features diagnosed at our

institution with a duplication on chromosome 17 including *TBCD* (bp 78,456,063-78,774,742; hg19) detected on illuminaCytoSNP-12v2.1 array, but no mutation was found.

Discussion

The *WDR62* mutation of patient 1 has not been previously described and is predicted to be a null allele. However this might not be sufficient to explain the severe phenotype as patient 6 (same mutation), is ambulant and has some socialization skills. Moreover, other patients described with a null mutation do not have such a severe neurological presentation (table 1)[5,6,10].

In addition to the *WDR62* mutation, patient 1 also has a change on both *TBCD* loci. It is possible, but not proven, that the severity in phenotype is due to the combination of *WDR62* and *TBCD* mutations. However, it is not clear at the moment whether the combination of duplication and the missense has a deleterious effect. Considering that both heterozygote parents are healthy, it is possible that, if *TBCD* mutations were pathogenic, their inheritance would be recessive[13]. In that case, the phenotypic variability seen within the *WDR62* spectrum could be explained by mutations in additional genes, such as *TBCD 11.* Alternatively, a single, heterozygote TBCD mutation could modify (and worsen) the effect of the WDR62 mutation.

Mutations in tubulin genes (*TUBA1A, TUBB2B, TUBB3, TUBB5, TUBA8*) and tubulin-associated proteins (*LIS1, DCX*) are increasingly recognized to cause cortical malformation[18,20]. Assembly of alpha/beta tubulin heterodimers is under control of chaperone proteins collectively called tubulin cofactors A-D[19]. Tubulin cofactor D (TBCD) is not only an essential factor in assembly but, under control of ADP ribosylation factor-like 2 (ARL2), in disassembly of microtubules and therefore plays a central role in cell motility[19,21]. Unique among tubulin cofactors, TBCD is implicated in centriologenesis, spindle organisation, cell abscission and ciliogenesis, at a crossroad between cell division, cell migration and signaling[15,16,21,22]. TBCD is highly expressed in brain and spinal cord, during foetal development and therefore predicted that mutations in any of the tubulin cofactors could be linked to disorders of cell motility and possibly to malformations of the cerebral cortex[19]. As mentioned, WDR62 protein is known to interact with mitotic spindles[8,9,10]. Its normal function might be strictly connected with a normal tubulin turnover. We did not

detect abnormal levels of *TBCD* mRNA in cultured fibroblasts; however a functional defect at the protein level cannot be excluded. Disruption of TBCD interaction can depend on subtle structural changes of interactors (as seen in *TUBB5* mutations[20]) and both over and underexpression of *TBCD* are known to be deleterious[16]. Possibly, an abnormal assembly of microtubules in the presence of *TBCD* mutations interferes with centriologenesis and spindle formation by WDR62. However, the phenotypic abnormalities of the (biallelic) *TBCD* mutation alone are not at present predictable.

The observation of the *TBCD* change was serendipitous in our patient because the array analysis was performed before the *WDR62* mutations were identified. However, molecular diagnosis of Mendelian traits is being replaced by targeted exome sequencing of panels of genes related to a specific phenotype[23]. If analysis of sequence variants remains limited to the known genes, the effect of non-allelic variants may go unnoticed. Triallelic and polygenic inheritance have been described in developmental disorders[24]. Our observation suggests that future application of exome sequencing to genetic diagnosis will require careful analysis of all detected variants for appropriate phenotype-genotype correlation predictions.

Acknowledgments

We thank the late Elly Verbeek for technical assistance and Dr. Frans W Verheijen for the useful discussions.

Table 1. Summary of the published mutations in WDR62 and where available the clinical details and brain imaging.Bold indicates clinical description in this report. Abbreviations: CSF:cerebrospinal fluid, CC:corpus callosum,. CT:Computerised Topography,GTC:generalised tonic clonic, ID: Intellectual Disability, MC:Microcephaly, MSG:Microcephaly with simplified gyration, MRI:Magnetic Resonance imaging, PMG:polymicrogyria.

Mutation (amino acid level)	Type mutation	Phenotype	Imaging	Reference
p.Glu536X	**Nonsense**	**Severe intellectual disability (ID), seizures, tetraparesis, microcephaly (MC)**	**Microlissencephaly**	**This paper (individual 1)**
p.Asp955Alafs*111	**Frameshift**	**Microcephaly, able to sit unsupported, co – operative, no speech, non-ambulatory**	**Simplified gyration with moderately thickened cortex**	**This paper (individual 2)**
p.Arg863His	**Missense**	**Microcephaly moderate developmental delay, insulin dependent diabetes**	**Symmetric bilateral polymicrogyria**	**This paper (individual 3)**
p.Asp955Alafs*111 p.Gly862Ser	Frameshift, missense	Microcephaly, infantile spasms, astigmatism, severe psychomotor delay and feeding difficulties	Frontal pachygyria; unilateral polymicrogyria	Müller et al, manuscript in preparation) (patient 4) (D6465)
p.Asp955Alafs*114	Frameshift	Microcephaly, no active speech	No gross cortical malformation	Müller et al, manuscript in preparation) (patient 5) (D6294)
p.Glu536X	Nonsense	Microcephaly at birth -2.7 SD, no seizures, intellectual disability, friendly behaviour, hyperactive, walked at 18 m, no language at 4y.	Pachygyria, corpus callosum dysgenesis	Müller et al, manuscript in preparation) (patient 6) (D6618)
p.Asp112Metfs*5	Frameshift	HC on 4th centile at 7 years. Walked at 4yr, at age of 7 could say a few words. Mild dysmorphic features with low sloping forehead	CT:Microcephaly with simplified gyral pattern (MSG)	Yu et al [5]
p.Asp112Metfs*5	Frameshift	Presented at 12 years unable to walk or talk, never sat or crawled or developed speech,GTC seizures at 9mth controlled with valproate. Mild dysmorphic features with low sloping forehead	MRI: MSG, enlarged lateral ventricles and relatively smaller right hemisphere	Yu et al [5]

p.Asp112Metfs*5	Frameshift	At 10 months HC -5.4SD. Early motor development normal, walking at 3yr no speech. Mild dysmorphic features with low sloping forehead,	MRI : MSG without ventricular enlargement, suggestion of subcortical heterotopias. Corpus callosum (CC) thin. Preserved brainstem and cerebellum	Yu et al [5]
p.Ser348Argfs*63	Splicesite	Microcephaly and no seizures	MRI: microcephaly cerebrum and diminised sulcation, bilateral band heterotopia in post frontal and parietal lobes and thin corpus callosum	Yu et al [5]
p.Val1313Glyfs*17	Frameshift	At birth HC -2.8SD, dysmorphic features: low sloping forehead, prominent occiput, broad and prominent nasal bridge, wide set eyes and fontanelle, Delayed development at 1 yr could sit, 18mt could move by sliding. Limited verbal expressions.	Polymicrogyria L>R, MSG on right abnormal CC. cerebellum and brain stem relatively preserved.	Yu et al [5]
p.Ser956Cysfs*38	Frameshift	HC at 2 months -5.3SD,high arched palate.	Thin CC, band hetertopias in frontal and parietal lobes	Yu et al [5]
p.Gly1280Alafs*21	Frameshift	MC noted at 6 months, walked at 1.5 yr, few words at 3yrs seizures at 1.5yrs	Microcephaly, relative sparing of brain stem and cerebellum, thin CC.	Yu et al [5]
p.Val65Met	Missense	Walked at 21 months,	MSG	Yu et al [5]
P.Val65Met	Missense	Spastic quadraparesis	Diffuse PMG and narrow right temporal parietal schizencephaly, dysmorphic corpus callosum	Yu et al [5]
p.Val65Met	Missense	Spastic quadraparesis	Diffuse PMG and right temporal openlip schizencephaly	Yu et al [5]
p.Val1402Glyfs*12	Frameshift	Ambulate at 2 yrs, few words, unable to feed herself	MC, cortical thickening, gray-white junction ill defined, diffuse pachygyria mainly affecting left hemisphere,brainstem and cerebellum grossly normal	Bilguvar et al [6]

p.Glu526X	Nonsense	Ambulate, at 4 years seizures, few words and understand simple commands at 6 years	Not published	Bilguvar et al [6]
p.Trp224Ser	Missense	Seizures at 2 yrs, 3yr –microcephaly, 1 word, ambulate with support, only speak one word. Severe ID	Diffuse polymicrogyria most visible in left posterior frontal lobe assoc with a schizencephalic cleft, cortical thickening in bilateral frontal lobes, hypoplasia of CC, atrophy of left side of brainstem,	Bilguvar et al [6]
p.Trp224Ser	Missense	3y – seizures, moderate ID, can ambulate and understand simple commands	Not published	Bilguvar et al [6]
p.Trp224Ser	Missense	MC, severe ID, can ambulate, seizures and self mutilating. More severely affected then his sister.	Not published	Bilguvar et al [6]
p.Gln470X	Nonsense	MC noted at one month, celiac, genu varum, severe ID,arachnodactly	Not published	Bilguvar et al [6]
p.Gly1280Alafs*21	Frameshift	MC, good head control and social smile at 10 years, has increased tone on examination.	Not published	Bilguvar et al [6]
p.Glu256Lys	Missense	3yr – MC, At 15 years microcephaly, prognathism, dysconjugate gaze, and dysarthria severe ID, can ambulate	Not published	Bilguvar et al [6]
p.Val1402Glyfs*12	Frameshift	20mt MC, developmental delay and severe ID	Microlissencephaly, hypoplastic CC	Bilguvar et al [6]
p.Val65Met	Missense		Simplified gyral pattern	Nicholas et al [8]
p.Arg438His	Missense	No clinical details apart from microcephaly and mild-moderate ID	No information	Nicholas et al [8]
p.Asp511Asn	Missense	“	No Information	Nicholas et al [8]
p.Asp511Asn	Missense	“	No information	Nicholas et al [8]
p.Ala1078Thr	Missense	“	No infomation	Nicholas et al [8]
p.Val1313Argfs*18	Frameshift	Severe ID, ambulatory	Simplified gyral pattern & cerebral cortex	Nicholas et al [8]

p.Met179fs*21	Frameshift	MC, motor milestones mildly delayed, speech grossly delayed, seizures 5yr	microcephaly pachygyria frontal cortex	Bhat et al [10]
p.Met179fs*21	Frameshift	MC, walked at 2yr, mild ID	microcephaly and dysplastic cortex involving left front middle region with widened sulcus	Bhat et al [10]
p.Cys300X	Nonsense	Mild MR, walked at 4yr, speech at 6y, seizures at 9yr	microcephaly, pachygyria, PMG bilaterally and microlissencephaly and band heterotopis bilaterally	Bhat et al [10]
p.Cys300X	Nonsense	Mild MR, walked at 2yr	MC, pachygyria and PMG bilateral frontal cortex	Bhat et al {10]
p.Ser696Alafs*4/p.Gln918Glyfs*18	Frameshift	Globally delayed, infantile spasms at 4/12, 9yr spastic quadraparesis	Bilateral PMG	Murdock et al [11]
p.Ser696Alafs*4/p.Gln918Glyfs*18	Frameshift	Ambulatory, uses age appropriate language	Small left hemisphere, PMG, focus of hetertopia in the right parietal region	Murdock et al [11]
p.Glu400Lys	Missense	Mild ID, delayed motor development, delayed speech (few words at 8yr), no seizures	CT: microcephaly, pachygyria,	Bacino et al [25]
p.Glu400Lys	Missense	Sat at 13mt, crawled at 15mt	No brain imaging	Bacino et al [26]
p.Asp511Asn	Missense	MC at birth, MR		Kousar et al [26]
p.Val1313Argfs*18	Frameshift	No information		Kousar et al [26]
p.Arg438His	Missense	No information		Kousar et al [26]
p.Gln648X	Nonsense	Abnormal sleeping habits	Reduced volume of right cerebral hemisphere, prominent CSF space and ill-defined gyri and nuclei pattern	Kousar et al [26]
p.His381Profs*48	Frameshift	MC -6SD, delay in ambulation, few words, epilepsy	Dilated ventricles, hypoplasia of corpus callosum, schizencephaly	Memon et al [27]
p.His381Profs*48	Frameshift	MC -7SD		Memon et al [27]

Supplemental Material

Clinical Reports

Individual 1: The proband's antenatal history was uneventful apart from microcephaly revealed at 34 week scan. Her parents are consanguineous and of Turkish descent. An amniocentesis was performed which showed a normal karyotype. She was born by SVD at 38 weeks gestation with good APGAR scores. Her birth weight was 2.8kg and she was noted to be microcephalic (at age 3 weeks head circumference of 28.5 cm, -4SD) with an impalpable fontanelle, prominent occiput and extremely flat frontoparietal skull. She was also hypotonic with an absent stepping reflex. At 7 months, her head circumference -7 SD below expected, she was severely developmentally delayed and hypotonic with some movement of head and legs, but unable to roll. She was able to fix, follow and smile sporadically. She developed epilepsy, which responded well to valproate. At 2 years HC was -9 SD below expected, she was noted to have dysmorphic features such as hypertelorism, upslanted palpebral fissures, large ears, broad thumbs and a short neck and severe psychomotor impairment (figure 1A). At age 5 years a MRI head revealed simplified gyration, an apparently thickened cortex in the frontal areas, dysplastic corpus callosum with agenesis of the middle part and the splenium, normal myelination and normal cerebellum, compatible with a microcephaly agyria-pachygyria or microlissencephaly (figure 2A-C). By the age of 7 years she had developed persistent crying, was exhibiting behavioural outbursts where she pulls hair, hits and bites. At the age of 13 years she has severe mental and motor impairment and epilepsy; she is unable to sit unsupported or to hold objects or speak, she reacts by smiling, can eat and chew and developed severe scoliosis. All metabolic and infective screens were negative. Ophthalmological examination was normal.

Individual 2: This boy presented with microcephaly at birth and developed in time ID and a spastic tetraparesis and epilepsy. At the age of 13 years he is able to sit unsupported, is cooperative and is interested in toys, drools and cannot speak but can communicate by gestures (figure 1B). He has epilepsy, which responded to valproate. His brain imaging at the age of 6 years showed moderate simplified gyration, moderately thickened cortex, dysplastic splenium of the corpus callosum, normal cerebellum. (figure 2D-F).

Individual 3: Female infant born to consanguineous parents from Tunisia after a pregnancy complicated by maternal diabetes which was diagnosed in the 3rd trimester controlled by diet. At birth, weight was 3750g and head

circumference as -3SD of expected. Early developmental milestones were achieved when assessed at 15 months and she has remained ambulant. Speech started at 4 years of age and was limited to a few words. She presented with diabetic keto acidosis at 6 years and has been on insulin since. A thorough clinical examination showed a normal height but head circumference -4SD below expected. Facial features included a sloping forehead, small chin, prominent nose, upward slant of the eyelid, prominent lower lip, profuse drooling. (figure 1C). Assessment of language and motor development indicated a general level between 2.5- 3.5 years. Neurologic examination showed mild asymmetric deep tendon reflexes, no neurolgical signs, mild hypotonia and valgus feet. EEG was normal. Brain MRI showed symmetric bilateral polymicrogyria extended to the temporal, parietal and frontal lobes, with incomplete opercularization of frontal and temporal lobes, no white matter abnormalities. Normal cerebellum, optic chiasma and nerves.(figure 2G-I) A CGH-array analysis and a karyotype are normal, except for several large regions of homozygosity.

Materials and Methods

SNP array and Copy Number detection

DNA was hybridized to the GeneChip Mapping 250K NspI array (Affymetrix) according to the standard protocol. Genotype data analysis and copynumber detection were performed in Affymetrix Genotyping Console v.4.1.1 using the BRLMM algorithm. The obtained .CEL and .CHP files were used for copynumber detection in CNAG v3.0.

Sequencing analysis

Amplification reactions were performed in a total volume of 20 µl, containing 1x PCR buffer with Mg (Roche), 200 µM of each dNTP, 1 µM forward primer, 1 µM reverse primer, 0.1 units Fast Start Taq DNA polymerase (Roche) and 25 ng genomic DNA. PCR conditions: 5' 96^{0}C, 10 cycles of 30'' 96^{0}C, 30'' 68^{0}C (-1^{0}C/cycle), 60'' 72^{0}C, followed by 25 cycles of 30'' 96^{0}C, 30'' 58^{0}C, 60'' 72^{0}C and a final extension for 5' 72^{0}C.

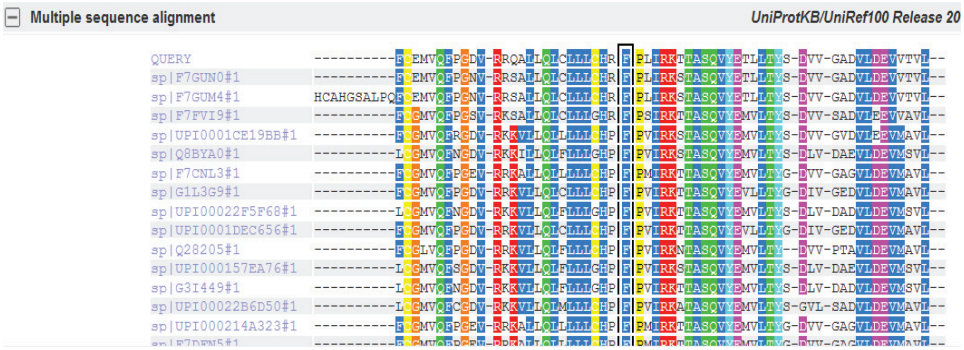
PCR reactions were purified with ExoSAP-IT (USB). Direct sequencing of both strands was performed using Big Dye Terminator chemistry ver. 3.1 (Applied Biosystems). DNA fragment analysis was performed using capillary electrophoresis on an ABI 3130 Genetic Analyzer (Applied Biosystems) and the software package Seqscape (Applied Biosystems, version 2.1).

Supplemental Table (S1)

Genes in duplication region on chromosome 17

Gene	Gene Symbol	Function
Forkhead box K2	FOXK2	Involved in regulation of viral and cellular promoter elements, not expressed in brain.
WDR45-Like	WDR45L	Unknown, not expressed in brain
Hypothetical LOC728694	LOC728694	Unknown
RAB40B	RAB40B	Member RAS oncogene family, not expressed in brain
Fructosamine 3 kinase related protein	FN3KRP	Intermediate metabolism, putatively involved in deglycation of proteins
Fructosamine 3 kinase	FN3K	Intermediate metabolism, deglycation of proteins
Zinc finger protein 750	ZNF750	Related to seborrhea-like dermatitis
Tubulin folding cofactor D	TBCD	Involved in microtubule dynamics, highly expressed in (foetal) brain and spinal cord
UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase-like	B3GNTL1	Unknown, not expressed in brain
Predicted LOC 644193	LOC644193	Unknown
Meteorin, glial cell differentiation regulator like	METRNL	Unknown, not expressed in brain
(no longer annotated on chrom 17q25)		
Similar to 60s ribosomal protein	FLJ43681	Pseudogene, non coding RNA

Figure S1. Protein Prediction Programme results for mutation c.3361T>G, in *TBCD* in Individual 1, also showing a snapshot of sequence conservation data generates by polyphen 2. This shows species conservation at amino acid position 1121 from across species with Rhesus Macaque at the top to the Three Spined Stickleback (bottom of the table).



SNAP analysis

```
Dear SNAP User,

Here are the results from your SNAP run

Result of SNAP prediction
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
Yana Bromberg and Burkhard Rost
NAR (2007)
_____________________________
#Query sequence  :  snapfasta
Including only predictions with:
         RI >= 0
        Expected Accuracy >= 50%

nsSNP        Prediction        Reliability Index        Expected Accuracy
-----        ------------        --------------------        --------------------
F1121V        Non-neutral                0                        58%
```

References

1. Mahmood S, Ahmad W, Hassah M.J (2011) Autosomal recessive primary microcephaly (MCPH): clinical manifestations,genetic heterogeneity and mutation continuum. Orphanet Journal or Rare Diseases 6:39e

2. Sajid Hussain M, Marriam Bakhtiar S, Farooq M, et al (2012) Genetic heterogeneity in Pakistani microcephaly families. Clin Gen. Epub Aug 7: 1-6.

3. Shen J, Eyaid W, Mochida G.H et al (2005) ASPM mutation identified in patients with primary microcephaly and seizures. J. Med. Genet. 42: 725-729, 2005

4. Thornton G.K, Woods C.G (2009) Primary microcephaly: do all roads lead to Rome? Trends Genet 25(11):501-10

5. Yu T.W, Mochida G.H, Tischfield D.J et al (2010) Mutations in WDR62,encoding a centrosome-associated protein, cause microcephaly with simplified gyri and abnormal cortical architecture. Nat Genet 42(11):1015-20

6. Bilguvar K, Kemel Ozturk A, Louvie A et al (2010) Whole exome sequencing identifies recessive WDR62 mutations in severe brain malformations. Nature 467(7312):207-10

7. Wasserman T, Katsenelson K, Daniliuc S et al (2010) A novel c-Jun N-terminal kinase (JNK)-binding protein WDR62 is recruited to stress granules and mediates a nonclassical JNK activation. Mol Biol Cell 21:117-130

8. Nicholaas A.K, Khurshid M, Desir J et al (2010) WDR62 is associated with the spindle pole and is mutated in human microcephaly. Nat Genet 42 (11):1010-1015

9. Bogoyevitch M.A, Yeap Y.Y.C, Zhengdong Q et al (2012) WDR40-repeat protein 62 is a JNK-phosphorylated spindle pole protein required for spindle maintenance and timely mitotic progression. J.Cell.Sci.125:5096-109

10. Bhat V, Girimaji S.C, Mohan G et al (2011) Mutations in *WDR62*, encoding a centrosomal and nuclear protein in Indian primary microcephaly families with cortical malformations. Clin Genet 80(6):532-40

11. Murdock D.R, Clark G.D, Bainbridge M.N et al (2011) Whole-Exome Sequencing Identifies Compound Heterozygous Mutations in *WDR62* in Siblings with Recurrent Polymicrogyria. Am J Med Gen 155A (9):2071-2076

12. Girirajan S, Jill A, Rosenfeld M.S., Coe B.P. et al (2012) Phenotypic heterogeneity of Genomic Disorders and rare Copy Number Variants. N.Engl.J.Med.367:1321-1331

13. McDonald D.M, Fahiminya S, Revil T et al (2010) Hemizygous mutations in SNAP29 unmask autosomal recessive conditions and contribute to atypical findings in patients with 22q11.2DS Am. J. Hum. Genet.50:80-90

14. Poulton C.J, Schot R, Kheradmand Kia S et al (2011) Microcephaly with Simplified Gyration, Epilepsy, and Infantile Diabetes Linked to Inappropriate Apoptosis of Neural Progenitors. Am.J.Hum.Gen 89:265-276

15. Lopez-Fanarraga M., Bellido J, Jaen C et al (2010) TBCD Links Centriologenesis, Spindle Microtubule Dynamics, and Midbody Abscission in Human Cells. PLoS One. 5(1):e8846

17. Manzini C.M, Walsh C.A (2011) What disorders of cortical development tell us about the cortex:one plus one does not always make two. Curr Opin Genet Develop. 21:333-339

16. Lopez-Fanarraga M, Avila J, Guash A et al (2001) Review: Postchaperonin Tubulin Folding Cofactors and Their Role in Microtubule Dynamics. Journal of Structural Biology 135: 219-229

18. Barkovic J.A, Guerrini R, Kuzniecky R.I et al (2012) A developmental and genetic classification for malformations of cortical development:update 2012.Brain.135:1348-69

19. Lundin V.F, Leroux M.R, Stirling P.C. (2010)Quality control of cytoskeletal proteins and human disease. Trends.Biochem. Sci.35(5):288-297

20. Breuss M, Heng JI, Poirier K, et al (2012) Mutations in the β-Tubulin Gene TUBB5 Cause Microcephaly with Structural Brain Abnormalities Cell Rep. 2(6):1554-62

21. Tian G, Thomas S, Cowan N.J (2010) Effect of TBCD and Its Regulatory Interactor Arl2 on Tubulin and Microtubule Integrity. Cytoskeleton. 67:706-714

22. Lopez-Fanarraga M., Carranza G, Castaño R et al (2010) Emerging roles for tubulin folding cofactors at the centrosome. Commun & Integr Biol 3(4):306-308

23. Dixon-Salazr T.J, Silhavy J.L, Udpa N et al (2012) Exome Sequencing can improve Diagnosis and Alter Patient Management. Science Translational Medicine 4:138ra78

24. Katsanis N, Ansley S. J, Badano J L, et al (2001) Triallelic inheritance in Bardet-Biedl syndrome, amendelian recessive disorder. Science 293: 2256-2259

25. Bacino C.A, Luis A.A, Wiszniewska J et al (2011) WDR62 missense mutation in a Consanguinous Family with Primary Microcephaly. Am J Med Gen:158A:622-625.

26. Kousar R, Jawad Hussan M, Khan B et al (2011) Mutations in WDR62 gene in Pakistani families with autosomal recessive primary microcephaly. BMC Neurology:11:119

27. Memon M.M, Raza S.L, Basit S et al (2013) A novel WDR62 mutation causes primary microcephaly in a Pakastani family. Mol Biol Rep.40:1:591-5

Web Resources

Database of genomic variants : http://projects.tcag.ca/variation/

1000 genome project: http://www.1000genomes.org/

Polyphen2: http://genetics.bwh.harvard.edu/pph2/

SNAP: http://rostlab.org/services/snap/

Chapter 5

General Discussion

Discussion

Increased apoptosis as a mechanism in primary microcephaly

Primary or congenital microcephaly with a normal brain structure or simplified gyral pattern has long been thought to be the result of a decrease in the proliferation secondary to a reduction in the neuroprogenitor pool[1]. Programmed cell death or "apoptosis" is essential for normal brain development with the highest levels in the ventricular and subventricular zone[2]. This has been supported by animal studies, for example mice haplo insufficient for *Magoh* (Mos +/-) demonstrate a microcephaly secondary to an increase in neuronal apoptosis as well as a decrease in the neuroprogenitor population[3]. *Magoh* encodes an exon junction component which regulates neural stem cell division[3]. Conversely mice that are deficient for pro-apoptotic genes (casp 9) show an increase in brain size[4]. In humans none of the known genes for primary microcephaly (MCPH) are thought to cause an increase in apoptosis, almost all of them encode for proteins that are components of centrosomes or mitotic spindle and thus postulated to cause microcephaly from a decrease in the progenitor pool[5]. This thesis describes the potential role of apoptosis in disorders presenting with a congenital or "primary" microcephaly.

Apoptosis or "programmed cell death" can occur in every cell in the body; it is under complex genetic control and ends with the cell undergoing distinct morphological changes. This includes the cell shrinking and condensing, the cytoskeleton collapsing, the nuclear envelope disassembling, the nuclear DNA breaking down and finally the cell surface alters allowing the cell to be phagocytosed without releasing any toxins and damaging surrounding cells[6].

Apoptosis occurs by activation of a multi-factorial pathway that ends in a caspase cascade. Every cell in the body contains pro-caspases which need to be cleaved in order to become active. There are two types of caspases, initiator caspases (caspase 2,8,9,10) and effector caspases (caspase 3,6,7). Initiator pro-caspases are activated by binding to an adaptor protein, these active initiator caspases then cleave the effector caspases downstream within moments which amplifies the signal and causes cell death via the "caspase cascade". Apoptosis can be triggered

by extracellular (extrinsic apoptosis) or intracellular (intrinsic apoptosis) pathways. These include the end point of DNA repair pathway or unfolded protein response (UPR) in intrinsic apoptosis or extra cellular pathways such as the TNF pathway for the extrinsic apoptosis. The end point for both is the caspase cascade. BCL-2 and IAP proteins are important for control of the caspase cascade and the influence is at many levels of the cascade, but are the essential mediators of the intrinsic apoptosis pathway (figure 1).

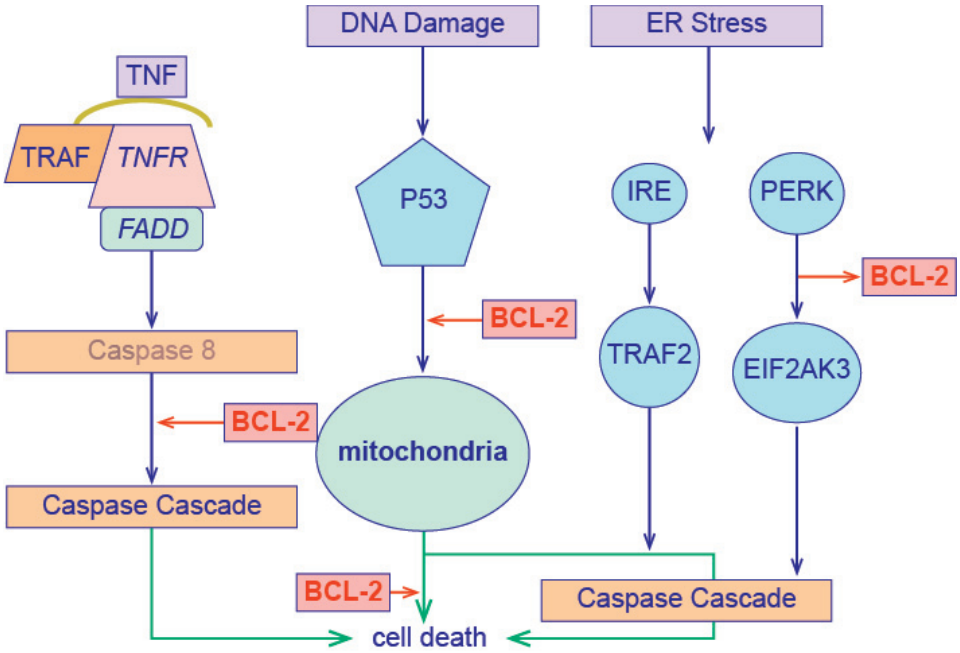


Figure 1. Simplified diagram of apoptotic pathways and showing the role of BCL-2 in all 3 pathways.

The intrinsic apoptosis pathway is considered to be essential in regulation of embryonic development and might be involved in the onset of microcephaly[7,8]. One of the intrinsic pathways leading to apoptosis is dependent on the endoplasmic reticulum (ER) unfolded protein response. Wolcott Rallison syndrome is a known example of apoptotic mechanism as cause of pancreatic and cerebral damage, caused by abnormal compensation of ER stress response[9]. We studied two families with an overlapping phenotype and we found mutations in immediate-

early-response-3-interacting protein-1(IER3IP1) being causative of Microcephaly Epilepsy Diabetes (MEDs) syndrome. Using tests for apoptosis in fibroblasts and post mortem data, which showed increased apoptosis in the cortex and pancreas we found evidence which supported this mechanism. Unfortunately very little is known about IER3IP1 only that it potentially interacts with immediate early response-3 (IER3). Both the mutations we described are in the predicted ER transmembrane domain of IER3IP1 which could explain the increased apoptosis observed is secondary to an inappropriate unfolded-protein response. IER3IP1 is reported to have a role in ceramide induced apoptosis[10] and appears from previous publications[11] and our experimental data to be under some control from TNF alpha. Thus this would place IER3IP1 in the caspase cascade. In support of this is in one individual harbouring an *IER3IP*1 mutation we found a dysregulation of *BCL-2* family, the anti-apoptotic *BCL-2* was down regulated by 120 fold and the pro-apoptotic *BCL-2A1* was up regulated by approximately 3.7 fold (previously unpublished data) along with dysregulation of genes involved in the TNF pathway, possibly placing IER3IP1 in both the extrinsic and intrinsic apoptotic pathway. (Table 1)

Table 1. Results of human apoptosis RT-pcr expression array (SABiosciences RT² Profiler PCR Arrays) showing dysregulation of apoptotic genes in patient 1 harbouring an *IER3IP1* mutation.

Gene	Fold up regulation	Gene	Fold down regulation
X1AP	12.1	*BCL.5-2*	120.6
BNIP	4.4	*CD40L*	16
DAPK1	19.64	*TRAF3*	9.9
BCL2A1	3.7	*TRAF4*	17.7
TNFRSF11B	4.2		
TP53	125.8		

The results from chapter 2 prompted a look to other disorders where primary microcephaly is a key feature with the possibility of apoptosis being a mechanism. This would include DNA repair disorders, in which microcephaly is an inconsistent feature[12]. Apoptosis versus degeneration of neural cells has been proposed as to why some DNA repair disorders present with microcephaly and others with brain atrophy and this is thought to be dependent on activation of the ATM/ATR pathway[12]. Individuals with Cerebro-Oculo-Facial-Skeletal (COFS) syndrome present

with severe congenital microcephaly, cataracts or microphthalmia, facial dysmorphism and arthrogryposis[13]. Brain MRI usually shows microcephaly with a simplified gyral pattern of the cerebral cortex, enlarged lateral ventricles and delayed myelination[14]. Individuals usually have severe neurodevelopmental delay and die in infancy secondary to brain stem dysfunction[13]. We tested several individuals who presented with COFS syndrome due to a mutation in *ERCC5* (XPG complementation group) for susceptibility to apoptosis and found a correlation between individuals who present with a prenatal onset of microcephaly and increased susceptibility of fibroblasts to apoptosis. Along with mouse studies of *Ercc5* knockout mice which showed a significant increase in apoptosis at week 4 of life in the cerebral cortex and have a phenotype of primary microcephaly with growth retardation. This provides circumstantial evidence for an increase in apoptosis being causative of primary microcephaly in COFS syndrome. Since microcephaly and growth retardation already occurs before birth, the most likely explanation of these abnormalities is the dysregulation of intrinsic developmental apoptosis. However, it is also possible that extrinsic apoptotic triggers play a role in disease progression and neurodegeneration.

ERCC5 is important in the DNA repair pathway and is responsible for the 3`incision in Nucleotide Excision Repair (NER) via the transcription coupled repair pathway (TCR). ERCC5 directly interacts with the other xeroderma pigmentosum-related (XP) proteins such as ERCC1 and ERCC4 which have been described as causative of COFS[15]. The NER pathway is important excision pathway for removing mutations derived from UV damage and protecting from cancer. The NER pathway is also postulated to have a role in repair outside the spectrum of UV damage as is evident by mutations in genes encoding proteins important in this pathway have a neurological phenotype[16]. ERCC5 may also have a role in base excision repair which has a role in repairing DNA damage after oxidative damage[17]. It has been proposed that severe mutations such as these seen in our COFS patients result in a neurological phenotype due to the defect in oxidative cell damage repair and substitutions result in a XP phenotype due to NER defect. Neurons are particularly susceptible to oxidative damage and accumulation of lesions leading to cell death could explain the progressive neurological phenotype exhibited by some patients[18].

ERCC5 has been reported to have a role as a negative regulator of apoptosis and this could be via the BCL proteins. BCL-X is one of the negative regulators of apoptosis and a key regulator in mammalian brain development[19] and it has been shown patient cells with ERCC5

null mutations have decreased levels of BCL-X, which would explain the increased sensitivity to apoptosis in patient cells[20].

Individuals with *PNKP* mutations, another DNA repair disorder described in chapter 3, also present with congenital microcephaly. This was original described by Shen et al as an epileptic encephalopathy syndrome (MCSZ), in individuals with microcephaly, intractable seizures and developmental delay[21]. We used whole genome sequencing (WGS) to explain the phenotype in our family to include primary microcephaly, intellectual disability, progressive cerebellar atrophy, severe polyneuropathy and seizures and, surprisingly, discovered a previously described homozygous mutation. PNKP is involved in single strand break (SSB) DNA repair by binding to a scaffold provided by XRCC1 and processes the repaired strands for the DNA ligase LIG3[22,23]. PNKP interacts with aprataxin protein, APTX (mutated in ataxia-oculomotor apraxia type 1) and with tyrosyl-DNA phosphodiesterase TDP1, which is mutated in spino-cerebellar ataxia - neuropathy type 1 (SCAN-1) which has a phenotypic overlap with our family[24.] This knowledge together with information from the animal models, (*XRCC1* KO mice show loss of cerebellar purkinje cells, ataxia and seizures[25]) can help to explain the cerebellar changes and neuropathy in our family. However, these additional phenotypes did not explain the congenital microcephaly seen in our patients and the patients described by Shen at al[21]. PNKP is also involved in the repair double strand break (DSB) related to non-homologus end joining (NHEJ) occurring during mitosis[23]. DSB is particularly important in dividing cells during development. In this pathway PNKP directly interacts (via the third fork head domain) with the scaffold XRCC4 and activates LIG4 (mutations cause severe immune deficiency with microcephaly)[23]. PNKP is activated and recruited by ATM and ATR which are associated with ataxia-telangiectasia and Seckel syndrome (primary microcephaly and primordial dwarfism) respectively. DNA ligase 4 (*Lig4*) knock out mice show increased apoptosis throughout the nervous system and die in the embryonic period. *Lig4* cerebral conditional KO mice (*Lig4* Nes-Cre) have a pronounced microcephaly and show increased apoptosis from E13.5, peaking at E15 via an ATM dependent and an ATM independent process[26]. Additionally PNKP interacts directly with ERCC4 to activate Lig4[27]. This could be used to predict that humans with the PNKP mutations would also exhibit a profound congenital microcephaly secondary to apoptosis during the developmental period. This had not been shown in humans in the original study[21]. In support of this hypothesis we did see an increased susceptibility to apoptosis in fibroblasts from individuals with PNKP mutation in comparison to controls.

Although it appears that the MCPH genes and the DNA repair genes are completely separate causes of microcephaly there is a link via MCPH1. MCPH1 was the first primary microcephaly gene identified and is associated with a small brain with a normal cortical architecture[28]. MCPH1 contains 3 BRCT (breast cancer C-terminal) motifs which are commonly found in DNA damage response proteins in order to ensure efficient cell cycle checkpoint arrest after DNA damage[28]. MCPH1 can modulate both the ATM and ATR repair pathway and its absence means that numerous proteins associated with the pathway fails to co-localise[29,30]. Interestingly disorders that are due to a defective ATR pathway (Seckel syndrome) also show microcephaly as a predominant feature.

It does appear that although apoptosis occurs in every cell type in the body, increased susceptibility to apoptosis results in a neurological phenotype. This is probably due to a combination of factors including differences in gene expression in different tissues and the susceptibilty of developing neurons to apoptosis. In the cases described in chapter 2, *IER3IP1* mutations result in a phenotype of MSG and diabetes, *IER3IP1* has a higher expression in the brain and pancreas therefore explaining this phenotype. DNA repair is important in all cells, but neurons appear to be particularly susceptible to damage, particularly oxidative damage and this could explain the predominance for a neurological phenotype as described in chapter 3. In summary, we have reviewed several distinct disorders where primary microcephaly appears to be related to increase in apoptosis in neurons in the pre-natal period, underscoring the primary role of apoptosis in brain development. DNA repair pathways and ER stress response appear to be key regulators of this pathway.

Potential for Prevention and Therapeutic Interventions

In chapter 4 we have described a patient with extreme microcephaly and changes at two loci. The first is WDR62, which is the MCPH2 gene related to primary microcephaly. The second TBCD has not been related to human disease. Based on the type of changes we observed in TBCD we cannot conclude whether there is a gain or loss of function. As over and under expression of TBCD has been shown to cause defect in microtubules[31], probably developmental defects could result from either. The putative involvement of TBCD raises interesting questions about potential intervention.

There is a link between primary microcephaly proteins and other diseases, specifically cancer. Cancer can be described as a disorder of proliferation

which requires increased mitosis and rapid turnover of the mitotic spindle or a decrease in apoptosis causing an overgrowth. Primary microcephaly proteins have been suggested as potential targets for cancer therapy because almost all of the genes encode for proteins involved in centrosome or mitotic spindle function. Disrupted spindle function is a target of some anti tumour drugs such as vinblastine and paclitaxel which are specific tubulin binding agents. These work to promote disassembly of the microtubule and arrest cell division[32,33]. Bisphosphonates are trialled against breast cancer work by causing up regulation of TBCB and cause disruption of microtubules[34]. So potentially other tubulin folding cofactors such as TBCD could be an antitumor target. This has the advantage of being tubulin specific and upregulation could cause microtubule release from the centrosome and G1 arrest[34], thus halting uncontrolled proliferation in cancer cells. Eventually pharmacological modulation of TBCD might also have an effect on individuals with primary microcephaly.

MCPH genes have been shown to be up regulated in cancer cell lines. *ASPM* (MCPH5) expression is increased in uterine and ovarian cancer cell lines when compared to normal tissue[35]. *STIL* (MCPH7) is upregulated in many cancer cell lines[36]. Decreasing the expression of STIL in a cancer cell line leads to increased apoptosis of the cancer cells secondary to delayed Cdk1 activation, which leads to the suggestion of STIL as a potential cancer target[36]. Potentially, as the gene mutations we have described lead to increased apoptosis, it could theoretically be possible to manipulate expression and induce apoptosis in cancer cell lines. It would be interesting to explore if incidence of cancer is lower in MCPH patients, as it has been suggested by anecdotal reports[37].

Brain damage by hypoxic insult either in neonatal hypoxic encephalopathy or by ischemic stroke occurs via an increase in apoptosis of neurons causing lasting and irreversible consequences. In neonatal hypoxic ischemic encephalopathy (HIE), the neonatal brain is sensitive to undergoing apoptosis and occurs days after the initial insult[38]. Rat models of HIE have shown high levels of caspase 3 that remain elevated for seven days after the initial injury[39]. Apoptosis has a prolonged role in the neurodegeneration after hypoxic ischemia in the newborn rat[39]. Anti-apoptotic gene BCL-2 has been suggested as a potential therapeutic target for neonatal HIE and there have been reports of animal experiments using adenovirus vectors to increase BCL-2 expression and thus decrease apoptosis both via caspase dependent and caspase independent pathways[38]. In view of our data generated showing decreased BCL-2 expression in our individual with an *IER3IP1* mutation (table 1) this would worth exploring to see if the

disease process could be arrested by increasing BCL-2 expression and possibly give some symptomatic relief for the seizures, neurodegeneration and in the case of IER3IP1, the diabetes which was difficult to control.

Concluding remarks

Since the human genome sequencing project (HGP) was completed in 2001 there has been rapid and continuous development in sequencing techniques, from Sanger sequencing that was developed in the 1970s to genomic SNP-based microarrays, to whole exome sequencing (WES) and whole genome sequencing (WGS) or “next generation sequencing”. It is now possible to sequence the whole genome of an individual in a few days. The use of this ‘state of the art’ technology for genome analysis presented in this thesis has led to important conclusions. We have identified the cause of a severe congenital microcephaly in *IER3IP1* mutations and, together with the observations in DNA repair syndromes with microcephaly, we confirmed the assumption that apoptosis can play a major role in the onset primary microcephaly. The use of even more advanced and unbiased technologies, such as WGS, has also led to two additional conclusions. First we identified a new phenotype linked to a known gene for microcephaly (*PNKP*, see chapter 3). Secondly, we identified a second gene, *TBCD*, as potential modifier of a phenotypic variable disorder such as *WDR62*-related microcephaly. Both these observation are paradigmatic to what the future of genetic analysis for rare Mendelian disorders will be regarding the use of such techniques. We may be able to diagnose new disorders caused by mutations in known genes and we may find additional mutations in the same individual that contribute and modulate the phenotype. However, a challenge will be to identify which mutations are clinically relevant. This will involve laboratory, animal and possibly stem cell research and this information may change over time. The concept that NGS is a “one time” test is probably misleading. Due to the rapid discovery of disease causing mutations the data generated from an individual could need to be re-analysed regularly depending on recent discoveries. This then brings up both ethical and practical questions of where the data should be stored and who should have access to it? These questions need to be addressed before we can use WGS regularly in a clinical diagnostic setting.

It is certain that medical specialists in several disciplines will need to be familiar with new techniques such as whole genome sequencing and collaborate with the clinical geneticists and basic scientists to further identify genes responsible andincrease our understanding of brain development

and disease processes. We then need to use this knowledge to perform translational research that can provide direct benefit to the individuals and families affected. In finding the genes responsible we can help to give the families some understanding, as the disease processes in the individuals described are unrelenting and lead to an early death. We can also offer prenatal testing, if the family requires it. As these diseases are rare and therefore the numbers of affected individuals are small, knowledge and multi-disciplinary skill base should be concentrated in specialized centres with scientific interest so that the insight into disease mechanisms creates new possibilities for intervention.

References

1. Barkovich AJ, Guerrini R, Kuzniecky RI,Jackson GD, Dobyns WB (2012) A developmental and genetic classification for malformations of cortical development: update 2012. Brain.135:1348-69

2. K Roth, C D`Sa (2001) Apoptosis and Brain Development, Mental Retardation and Developmental Disabilities.2001.Research Reviews. 7:261-266

3. Silver DL, Watkins-Chow DE, Schrech KC, Pierfelice TJ, Larson DM, Burnetti A, Jm Liaw HJ, Myung K, Walsh CA, Gaiano N, et al (2010) The exon junction complex component Magoh controls brain size by regulating neural stem cell division. Nat. Neurosci. 13:551-558

4. Kuida K, Haydar TF, Kuan CY, Taya C, Karasuyama H, S-S Su M, Rakic P, Flavell RA (1998) Reduced Apoptosis and Cytochrome c-Mediated Caspase Activation in Mice lacking Caspase 9. Cell. 94 :325-337.

5. Mahmood S, Ahmad W, Muhammad H (2012) Autosomal recessive primary microcephaly (MCPH): clinical manifestations, genetic, heterogeneity and mutation continuum.Orphanet Journal of Rare Diseases.6:39

6. Alberts B, Johnson A, Lewis J, Raff M, Roberts K, Walter P (2008) Molecular Biology of the Cell. 5th Edition. Garland Science

7. Zimmermann KC, Green DR (2001) How cells die: apoptosis pathways. J Allergy Clin Immunol .108:s99-103

8. Galluzzi L, Vitale I,Abrams JM, Alnemri5 ES, Baehrecke EH,Blagosklonny MV, Dawson8 TM, Dawson8 VL, El-Deiry WS (2012) Molecular definitions of cell death subroutines: recommendations of the Nomenclature Committee on Cell Death 2012. Cell Death and Differentiation .19:107-120

9. de Wit MCY, de Coo IFM, Julier C, Delephine M, Lequin MH, van de Laar M, Sibbles BJ, Bruining GJ, Mancini GMS (2006) Microcephaly and simplified gyral pattern of the brain associated with early onset insulin-dependent diabetes mellitus. Neurogenetics 7:259-63

10. Decraene C, Brugg B, Ruberg M, Eveno E, Matingou C, Tahi F, Mariani, J, Auffray C, Pietu G (2002) Identification of genes involved in ceramide-dependent neuronal apoptosis using cDNA arrays. Genome Biol. 3: 1-22

11. Yiu WH, Yeung TL, Poon JWM, Tsui SKW, Fung KP, Waye MMY (2010) Transcriptional regulation of IER3IP1 gene by tumor necrosis factor-alpha and Sp family proteins. Cell Biochem Funct 28:31-37

12. O`Driscoll M, Jeggo PA (2008) The Role of the DNA damage response pathways in brain development and Microcephaly: Insight from human disorders. DNA Repair.7:1039-1050

13. Natale V (2010) A Comprehensive Description of the Severity Groups in Cockayne Syndrome. Am J Med Genet. 155A:1081-95

14. Koob M, Laugel L, Durand M, Fothergill H, Dalloz H, Sauvanaud H, Dollfus H, Namer IJ, Dietemann JL (2010) Neuroimaging in Cockayne Syndrome. Am.J.Neuroradiol. 31:1623-30

15. de Boer J, Hoeijmakers JH (2000) Nucloetide excision repair and human syndromes. 21: 453-460

16. McKinnon P (2009) DNA repair Deficiency and Neurological Disease. Nat Rev Neurosci.10:100-112

17. Wang JY, Sarker AH, Cooper PK, Volkert MR (2004) The single-strand DNA binding activity of human PC4 prevents mutagenesis and killing by oxidative damage. Mol Cell Biol.24:6084-6093

18. Soltys DT, Rocha CRR, Lerner LK, de Souza TA, Munford V, Cabral F, Nardo T, Stefanini M, Sarasin A, Cabrel-Neto JB, Menck FM (2013) Novel *XPG (ERCC5)* mutations affect DNA repair and cell survival under ultraviolet but not oxidative stress. Hum Mut. DOI:10.1002/humu.22259

19. Kuan CY, Roth KA, Flavell RA, Rakic P (2000) Mechanism of programmed cell death in the developing brain. Trends Neurosci. 23:287-293.

20. Lomonaco SL, Xu XS, Wang G (2009) The role of Bcl-x(L) protein in nucleotide excision repair-facilitated cell protection against cisplatin-induced apoptosis. DNA Cell Biol.28(6):285-94.

21. Shen J, Gilmore EC, Marshall CA, et al (2010) Mutations in PNKP cause microcephaly, seizures and defects in DNA repair. Nat Genet 42:245-249

22. Caldecott KW (2008) Single-strand break repair and genetic disease. Nat Rev Genet 9:619-631

23. Weinfeld M, Mani RS, Abdou I, Aceytuno RD, Glover JN (2011) Tidying up loose ends: the role of polynucleotide kinase/phosphatase in DNA strand break repair. Trends Biochem Sci 36:262-271

24. Plo I, Liao ZY, Barcelo JM, Kohlhagen G, Caldecott KW, Weinfeld M, Pommier Y (2003) Association of XRCC1 and tyrosyl DNA phosphodiesterase (Tdp1) for the repair of topoisomerase I-mediated DNA lesions. DNA Repair 2:1087-1100

25. Lee Y, Katyal S, Li Y, El-Khamisy SF, Russell HR, Caldecott KW, McKinnon PJ (2009) The genesis of cerebellar interneurons and the prevention of neural DNA damage require XRCC1. Nat Neurosci. 12:973-80.

26. Gatz SA, Ju L, Gruber R, et al (2011) Requirement for DNA ligase IV during embryonic neuronal development. J Neurosci 31:10088-10100

27. Mani RS, Yu Y, Fang S, Lu M, Fanta M, Zolner AE, Tahbaz N, Ramsden DA, Litchfield DW, Lees-Miller SP, Weinfeld M (2010) Dual modes of interaction between XRCC4 and polynucleotide kinase/phosphatase: implications for nonhomologous end joining. J Biol Chem. 285:37619-37629

28. Jackson A P, Eastwood H, Bell SM, Adu J, Toomes C, Carr IM, Roberts E, Hampshire DJ, Crow YJ, , Mighell AJ, Karbani G, Jafri H, Rashid Y, Mueller R F, Markham A F, Woods CG. (2002) Identification of microcephalin, a protein implicated in determining the size of the human brain. Am. J. Hum. Genet 71: 136-142

29. Lin S.-Y, Rai R, Li K, Xu Z-X, Elledge S J (2005) BRIT1/MCPH1 is a DNA damage responsive protein that regulates the Brca1-Chk1 pathway, implicating checkpoint dysfunction in microcephaly. Proc. Nat. Acad. Sci. 102:15105-15109

30. O`Driscoll M, Jackson AP, Jeggo PA (2006) A Causal Link Between Impaired Damage Response Signalling and Microcephaly. Cell Cycle 5:20:2239-2344

31. Lopez-Fanarraga M, Avila J, Guash A,Coll M, Zabala JC. (2001) Review: Postchaperonin Tubulin Folding Cofactors and Their Role in Microtubule Dynamics. Journal of Structural Biology 135: 219-229

32. Lundin VF, Leroux M, Stirling P (2010). Quality control of cytoskeletal proteins and human disease. Cell Press 35:288-97

33. Lin YF Tsai WP, Liu HG, Liang PH (2009) Intracellular beta-tubulin/ chaperonin containing TCP1-beta complex serves as a novel chemotherapeutic target against drug resistant tumours. Cancer Res.69;6879-6888

34. Bivi N Bivi N, Romanello M, Harrison R, Clarke I, Hoyle DC, Moro L, Ortolani F, Bonetti A, Quadrifoglio F, Tell G, Delneri D.(2009) Identification of secondary targets of N-containing bisphosphonates in mammalian cells via parallel competition analysis of the barcoded yeast deletion collection. Genome Biol 10:R93

35. Kouprina N, Pavlicek A, Collins NK, Nakano M, Noskov VN, Ohzeki J, Mochida GH, Risinger JI, Goldsmith P, Gunsior M, Solomon G, Gersch W, Kim JH, Barrett JC, Walsh CA, Jurka J, Masumoto H, Larionov V (2005) The microcephaly ASPM gene is expressed in proliferating tissues and encodes for a mitotic spindle protein. Hum Mol Genet.14:2155-65.

36. Erez A, Castiel A, Trakhtenbrot L, Perelman M, Rosenthal E, Goldstein I, Stettner N, Harmelin A, Eldar-Finkelman H, Campaner S, Kirsch I, Izraeli S.(2007) The SIL gene is essential for mitotic entry and survival of cancer cells. Cancer Res. 67:4022-7

37. Thornton GK, Woods CG. Primary microcephaly: do all roads lead to Rome? (2009)Trends Genet.25:11:501-10

38. Fatemi A, Wilson MA, Johnston MV (2009) Hypoxic-ischemic encephalopathy in the term infant Clin Perinatol. 36:835-58

39. Nakajima W, Ishida A, Lange MS, Gabrielson KL, Wilson MA, Martin LJ, Blue ME, Johnston MV (2000) Apoptosis has a prolonged role in the neurodegeneration after hypoxic ischemia in the newborn rat. J Neurosci 20:7994-8004

Addendum

Summary

Chapter 1, the introduction describes the definition of microcephaly, a brief overview of potential role of microcephaly genes in evolution and a summary of current knowledge of genetic caused of congenital microcephaly.

Chapter 2 describes two families whose children were affected by a combination of microcephaly, epilepsy, neonatal diabetes and early death. By using homozygosity mapping and linkage analysis we identify homozygous *IER3IP1* mutations in the affected individuals from both families. Using evidence from post mortem data and from fibroblasts from an affected individual we show a link between increased susceptibility to apoptosis and description of a new syndrome Microcephaly, Epilepsy and Diabetes (MEDs, OMIM 614231).

Chapter 3 is focused on the link between DNA repair disorders and microcephaly. In **chapter 3.1** we use whole genome sequencing to identify *PNKP* mutations in a family causing a combination of primary microcephaly, progressive polyneuropathy, severe progressive cerebellar atrophy, mild epilepsy and intellectual disability which expanded the known phenotype. We showed that patient cells have an increased tendency to apoptosis under stress conditions which could help to explain the microcephalic phenotype. These findings led us to look at other DNA repair disorders that present with a primary microcephaly in order to explore a potential link between apoptosis and microcephaly. In **chapter 3.2** we describe the clinical presentation and studied the tendency to apoptosis in fibroblasts derived from eight individuals bearing *ERCC5 (XPG)* mutations with variable presentation, ranging from mild neurologic symptoms to severe microcephaly and death in early infancy and one Cockayne syndrome patient. We found that apoptotic levels correlated with the severity of microcephaly and disease course. This link was supported by data from *Ercc5* knock-out mice which showed increased apoptosis at 4 weeks in tissues including frontal cortex.

Chapter 4 describes individuals with *WDR62* mutations and one severely affected individual who as well as a homozygous mutation in *WDR62*, has duplication on chromosome 17 and a predicted pathogenic mutation in *tubulin co-factor D (TBCD).* We explore the potential role as a phenotypic modifier of *TBCD* and the potential role of polygenetic inheritance in the presentation of microcephaly associated with cortical malformations.

The thesis ends at **chapter 5, the discussion** with an overview of our findings and possible future developments. We have showed a link between apoptosis and microcephaly in human disease and discuss the relevance in the clinical setting including future possible interventions.

Samenvatting

Hoofdstuk 1, de introductie, beschrijft de definitie van microcefalie, een kort overzicht van de potentiële rol van microcefalie genen in de evolutie en een samenvatting van de huidige kennis van genetische oorzaken van aangeboren microcefalie.

In **hoofdstuk 2** worden 2 families beschreven, waarvan de kinderen een combinatie hebben van microcefalie, epilepsie, neonatale diabetes en vroegtijdig overlijden. Met behulp van homozygotie mapping en koppelingsonderzoek zijn homozygote *IER3IP1* mutaties gevonden in de aangedane individuen van beide familes. Met behulp van bewijs verkregen uit *post mortem* bevindingen en fibroblasten van een aangedaan individu konden we een verband beschrijven tussen een verhoogde vatbaarheid voor apoptose en microcephalie. Dit werk heeft geleidt tot de beschrijving van een nieuw syndroom: Microcefalie, Epilepsie en Diabetes (MED, OMIM 614231).

Hoofdstuk 3 is gericht op het verband tussen DNA repair aandoeningen en microcefalie. In **hoofdstuk 3.1** beschrijven we het gebruik van whole genome sequencing om *PNKP* mutaties te identificeren in een familie met een combinatie van primaire microcefalie, progressieve polyneuropathie, ernstige progressieve cerebellaire atrofie, milde epilepsie en intelectual disability , wat heeft geleid tot een uitbreiding van het reeds beschreven fenotype. We hebben laten zien dat fibroblasten van patiënten een verhoogde vatbaarheid voor apoptose hebben onder stress- omstandigheden, dat kan helpen om het fenotype met microcephalie te verklaren. Dit was voor ons de aanleiding om andere DNA repair aandoeningen te bestuderen die zich presenteren met een primaire microcefalie, om zo een potentiëel verband te leggen tussen apoptose en microcefalie. In **hoofdstuk 3.2** beschrijven we de klinische presentatie en bestudeerden we de vatbaarheid voor apoptose in fibroblasten verkregen van acht individuen met *ERCC5* (*XPG*) mutaties met variabele presentatie, variërend van milde neurologische symptomen tot ernstige microcefalie en overlijden op vroege kinderleeftijd en een patiënt met Cockayne syndroom. We vonden dat de mate van apoptose correleerde met de ernst van de microcefalie en het ziekteverloop. Dit verband werd ondersteund door beschrijvingen van *Ercc5* knock-out muizen, die op de leeftijd van 4 weken verhoogde apoptose laten zien in weefsel, waaronder de frontale cortex.

Hoofdstuk 4 beschrijft individuen met *WDR62* mutaties en een ernstig aangedaan individu met een homozygote mutatie in *WDR62*, met een duplicatie op chromosoom 17 en een mutatie, voorspeld als pathogeen, in *tubulin co-factor D* (*TBCD*). We onderzoeken de mogelijke rol van *TBCD*

als een modulator van het fenotype en de potentiële rol van polygenetische overerving in de presentatie van microcefalie geassocieerd met corticale malformaties.

Het proefschift eindigt met **hoofdstuk 5**, de discussie, met een overzicht van onze bevindingen en mogelijke toekomstige ontwikkelingen. We hebben een verband laten zien tussen apoptose en microcephalie in humane ziekte en bespreken de relevantie voor de klinische setting, inclusief mogelijke behandelingsopties in de toekomst.

List of Abbreviations

AD	Autosommal Dominant
AR	Autosommal Recessive
BRCT	Breast cancer susceptibility protein C-terminal
CMV	Cytomegalovirus
CNS	Central Nervous System
CNV	Copy Number Variation
COFS	Cerebro-Ocular-Facial- Skeletal
CS	Cockayne Syndrome
CSF	Cerebrospinal fluid
CT	Computerised Tomography
DNA	Deoxyribonucleic acid
DSB	Double Stranded Break
DTT	Dithiothreitol
EEG	Electroencephalograph
EMG	Electromyogram
ER	Endoplasmic Reticulum
HIV	Human Immunodeficiency Virus
HMSN	Hereditary Motor Sensory Neuropathy
ID	Intellectual Disability
LMD	London Medical Database
LOH	Loss of Heterozygosity
MCPH	Microcephaly Primary
MC+PMG	Microcephaly and Polymicrogyria
MEF	Mouse Embryonic Fibroblasts

MLIS	Microcephaly and Lissencephaly
MRI	Magnetic Resonance Imaging
MSG	Microcephaly Simplified Gyration
MTOC	Microtubule Organising Centre
NER	Nucleotide Excision Repair
OFC	Occipito Frontal Circumference
PCH	Ponto-Cerebellar Hypoplasia
PCM	Pericentriolar Material
PKU	Phenyl Ketonuria
PND	Permanent Neonatal Diabetes
RNA	Ribonucleic Acid
SD	Standard Deviation
SNP	Single Nucleotide Polymorphism
SSB	Single Stranded Break
TCR	Transcription Coupled Repair
TNF	Tumour Necrosis Factor
TUNEL	TdT-mediated dUTP Nick-End Labeling
UPR	Unfolded Protein Response
UV	Ultraviolet
WES	Whole Exome Sequencing
WGS	Whole Genome Sequencing
WRS	Wolcott Rallison Syndrome
XP	Xeroderma Pigmentosum

Curriculum Vitae

The author was born on the 19-06-1979 in Yeovil, United Kingdom. She completed A Levels in Biology, Chemistry, Psychology and BTEC in Music Theory and Performance at Strode College in Somerset in 1997. She then started medical school at St Marys School of Medicine at Imperial College, London. She graduated in 2003 after receiving honours in Paediatrics and Obstetric and Gynaecology and an additional Bsc in Infection and Immunology. She entered paediatric training in London in 2004 and has gained experience in (among others) paediatric neuromuscular disease at the Hammersmith Hospital, London and paediatric neurology at Kings College Hospital London. She has obtained Membership of the Royal College of Paediatrics and Childhealth (MCPCH). In June 2008 she started her PhD training on the genetics of microcephaly at the department of Clinical Genetics of the Erasmus Medical Center under supervision of Dr. G. Mancini, which resulted in the present dissertation. Later this year she will take up a new post as a paediatric resident at Princess Margaret Hospital, Perth, Western Australia to further her interest in paediatric neurology.

She was Captain of Imperial College Equestrian Club in London and competed at Local and National level in dressage and show jumping. She has continued this interest in Rotterdam by competing in dressage on her shared horse “Bella”. She is married to Carl Schultz and has two children, Zachary aged 3 years and Evie aged 5 months.

List of Publications

1. **Poulton C.J**, Schot R, Kia S.K, Jones M, Verheijen F.W, Venselaar H, de Wit MC, de Graaff E, Bertoli-Avella A.M, Mancini G,M (2011) **Microcephaly with simplified gyration, epilepsym and infantile diabetes linked to inappropriate apoptosis of neural progenitors.** Am J Hum Genet.;89(2):265-76

2. Verbeek E, Meuwissen ME, Verheijen FW, Govaert PP, Licht DJ, Kuo D.S, **Poulton C.J**, Schot R, Lequin MH, Dudink J, Halley DJ, de Coo R.I, den Hollander J.C, Oegema R, Gould D.B, Mancini G.M (2012) **COL4A2 mutation associated with familial porencephaly and small-vessel disease**. Eur J Hum Genet;20(8):844-51

3. Kheradmand Kia S, Verbeek E, Engelen E, Schot R, Poot RA, de Coo IF, Lequin MH, **Poulton C.J**, Pourfarzad F, Grosveld FG, Brehm A, de Wit MC, Oegema R, Dobyns WB, Verheijen FW, Mancini GM (2012) **RTTN mutations link primary cilia function to organization of human cerebral cortex** . Am J Hum Genet.;91(3):533-40

4. **Poulton C.J**, Oegema R, Heijsman D, Hoogeboom J, Schot R, Stroink H, Willemsen MA, Verheijen FW, van de Spek P, Kremer A, Mancini GM (2013) **Progressive cerebellar atrophy and polyneuropath: expanding the spectrum of PNKP mutations.** Neurogenetics. 2013 Feb;14(1):43-51.

5. **Poulton C.J**, Schot R, Barnhoorn S, Vermij W.P, Verheijen F.W, Brooks A.S, van der Knaap M.S, Ausems M.G.E.M, Hoeijmakers J.H.J, Jaspers, Mancini G.M.S (2013) **Severe microcephalic Cerebro-Ocular-Facial-Skeletal (COFS) syndrome with ERCC5 mutation shows UV-independent increased susceptibility to apoptosis.** Manuscript Submitted.

6. **Poulton C.J**, Schot R, Seufert K, Lequin M.H, Accogli A, D`Annuzio G, Villard L, de Coo R.I, Catsman-Berrevoets, Grasshof U, Kattentidt A, Morris-Rossendahl D, Mancini G.M.S. (2013) **Severe presentation of *WDR62* mutations: is there a role for modifying genetic factors?** Manuscript submitted

PhD portfolio

Summary of PhD training and teaching

Name: Cathryn Poulton Erasmus MC Department: Clinical Genetics Research School: Medical genetics Centre (MGC), Southwest Netherlands	PhD Period: June '09 – June '13 Promoter: Prof.dr. R. Hofstra and Prof dr. F. Grosveld Co-promoter: Dr. G.M.Mancini		
1. PhD training	**Year**	**Workload (Hours)**	**Workload (ECTS)**
General courses			
– Classical Methods for Data-Analysis	2011		5.7 ECTS
– Molecular and Cell Biology	2009		6 ECTS
– SNPs and Human Disease	2008		1 ECTS
Specific courses			
– Biomedical Research Techniques	2008		1.5 ECTS
– From Development to Disease	2009		1.5 ECTS
– Biomedical Research Techniques	2009		0.8ECTS
– SPSS: Statistic Course for Novices	2009		1.5ECTS
– Advanced Paediatric Life Support	2012		1 ECTS
– BPNA Epilepsy Training Level 2	2012		1 ECTS
Seminars and Workshops			
– Ensemble Workshop	2008		0.8 ECTS
– Workshop on Photoshop and Illustrator CS4	2010		0.3 ECTS
– PhD Career Day	2011		
– Participated in working group at European Research Conference on Paediatric Neurology, Tubingen	2008		1 ECTS
Presentations			
– In the department (2/year)	2008-2013		
– CHD group weekly meetings	2008-2013		
(Inter) National Conferences			
– Genetica Retraite, Rolduc, (oral presentation)	2009		1 ECTS
– 14th Congress of the European Federation of Neurological Societies, oral presentation and winner of "Young Neurologist of the year	2010		1 ECTS
– European Human Genetics Conference, Amsterdam (oral presentation)	2011		1 ECTS
– Netherlands Human Genetic Society Conference (oral presentation)	2011		1 ECTS
– European Society of Magnetic Resonance in Neuropaediatrics (oral presentation)	2011		1 ECTS
– Netherlands Paediatric Neurology Society 2012 (oral presentation)	2012		1 ECTS

Others – Clinical Genetics work discussion – Group weekly meetings	 2009-2013 2009-2013	 200 400	
2. Teaching/Supervising			
– Teaching Medical Students – Mentoring Junior Doctors	2011-2012 2011-2012		1 ECTS 1 ECTS
Total			31.1 ECTS

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Vanessa, I am glad we met and have a mutual love of animals! Thank you for taking care of Percy, Noah and Hoover and for your friendship. Congratulations on your PhD and I know we will keep in touch.

Everyone in the lab and the department of genetics I was truly privileged to have worked with you and thank you.

Gabey (and Summer and Quinn), this is as soppy as you will ever get from me and it is in writing! So enjoy…..Without you I think my time in Rotterdam would have been very very different. A shared love of horses brought us together and especially your crazy mare, Bella who I would fall in love with as well. You have been so supportive and a true friend and you are a very special person. I feel privileged to have been part of your life and know and love your children since they were newborn. I know we will see each other regularly down under so I am not going to make this too sad, but thank you for being you.

Donna, we have so much in common and I am only sorry that we met towards the end of my stay in the Netherlands. Thank you for your messages of support especially over the last few months. I only hope I can be as supportive when you are at the same stage soon. Definitely a big pair of “mummy power pants” needed!

Renee and Archie thank you for coming to help with the children so I could spend 6 weeks writing, without you this thesis would definitely still be in the “ideas” phase or part of my infamous “job list”.

Christina and of course little Tamsin, thank you for your support especially when I was working late at night, (as you are a night owl!), I hope your book is the next one to be published! I am very proud of my little sister and know you will continue to be successful.

Mum and Dad, you have always made me feel that I can achieve anything I wanted if I worked hard enough. “I can`t” was just not said in our house as we were growing up. Thank you for installing in me faith in myself and a good work ethic to help me achieve the very best I can. Less than 100% in tests was always answered with “what happened to the 3%” ensuring that we knew we should always strive to better ourselves. Daddy I hope you will see me as a “real Doctor” and as clever as you! I know now how hard it must be to encourage your child without being over bearing and you two did a perfect job of it!

Carl, the love of my life and the man who helps me be the person I want to be. Thank you for persuading me to come to Holland to work. I had my doubts, mostly because what is the point of moving somewhere with the exact same or possibly even worse weather then England? But as usual you proved to be right. I have learned a huge amount about genetics, neurology, and science but also about how to move to a different country, make new friends and fit in. You have always been supportive, despite any

personal misgivings and for that I thank you. I know we are a great team and are stronger together.

Zachary and Evie, my two babies who I love more than life itself: I would do anything for either of you. Both of you will forever have links to Rotterdam as it states on your passport you were born here. Zachary now you are 3 years old you will remember some of your time in Holland. I hope that you will remember you had lots of laughter, lots of friends and the sledging on the frozen lakes. You are such a strong little boy who is capable of such cheekiness and kindness. I love watching you discover and learn and that you say with such pride “I am English but I can speak Nederlands as well”. Evie, you came along just after Christmas last year and I fear your earliest memories will be of your mummy feeding you while typing on a computer; you must also win the record for the youngest person to be in a genetics work meeting! You are such a good baby and so happy and I am enjoying already watching you start to notice and interact with the world around you. To Zachary and Evie thank you for choosing me to be your mummy, you make everything worthwhile and I hope you will feel that you can also achieve whatever you desire without limitations as you grow up.

We have great adventures planned for the next few years so onwards to the next chapter.