Identification and Characterisation of the Gene for Börjeson-Forssman-Lehmann Syndrome

By

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Amendments

Pg 11, line 5.
The reference for Encylopáedia Brittanica is http://www.britannica.com/.

Pg 13, chapter 1.3.
The sentence should be rephrased as the following.
Mental retardation encompasses a heterogeneous group of disorders. A great deal of effort has gone into determining both environmental and genetic causes in the general population.

Pg 16, chapter 1.4.1, line 9.
The sentence should read "However, as the genes responsible for these disorders began to be identified, the initial descriptions of the disorders often changed, …".

Pg 20, chapter 1.4.3, line 10.
Eponomously should be spelt eponymously.

Pg 22, table 2.
MeCP2 function is best described as a transcription repressor.

Pg 23, table 2.
Incontentia should be spelt incontinentia.

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Thesis Summary

Mental retardation (MR) affects approximately 2-3% of the population. A high proportion of cases is due to genetic factors, with estimates of approximately 25% of MR being caused by genes on the X chromosome.

One of the earliest X-linked forms of MR described was Börjeson-Forssman-Lehmann syndrome (BFLS; MIM 301900). Affected males display a phenotype of mild to severe MR, gynecomastia, hypoplastic external genitalia, obesity, deep set eyes, visual problems, "heavy" face, long ears (specifically earlobes), shortened toes and tapered fingers, with variable features including epilepsy, microcephaly and short stature.

The gene for BFLS was known to map to a large region on Xq26-q27; however, the molecular basis of BFLS remained unknown. This research project refined the localisation of the BFLS gene, identified the gene, and completed preliminary analysis of the cellular function of the protein.

The critical genetic interval was reduced from 24.6 cM to approximately 8 cM, and an *in silico* physical and transcriptional map of this reduced minimal BFLS region was constructed. Of the 62 identified genes, 19 were screened for mutations.

Mutations associated with BFLS were identified in a novel PHD-like zinc finger gene, which has since been named *PHF6*. The full genomic structure, expression analysis in both human tissues and mouse brain, and cellular localisation of the protein was analysed. Eight different missense and truncation mutations were identified in seven familial and two sporadic cases of BFLS. *PHF6* is a widely expressed gene, present in nearly all adult tissues studied, with specific developmental stage expression in mouse brain. Transient transfection studies with tagged PHF6 protein showed diffuse nuclear staining with prominent nucleolar

accumulation. Such localisation, combined with the presence of two PHD-like zinc fingers, is suggestive of a role for PHF6 in transcription.

This work facilitates precise and early diagnosis of individuals affected with BFLS. Families will benefit from a direct DNA test of carrier status for females, and with the aid of counselling will have the ability to make informed reproductive choices. The identification of this gene also provides wider insight into the cellular pathways that are integral for normal cognitive function and physical development.

Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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19th April 2003

Karen Marie Lower Date

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It's all swings and roundabouts

List of Abbreviations

AGTR2	angiotensin II receptor, type 2
ARHGEF6	Rho guanine nucleotide exchange factor 6
ARX	aristaless-related homeobox, X-linked
ATR-X	alpha-thalassemia/mental retardation syndrome, X-linked
BFLS	Börjeson-Forssman-Lehmann Syndrome
cM	centimorgans
FGD1	faciogenital dysplasia gene 1
FHF2	fibroblast growth factor homologous factor 2
FMR1	fragile X mental retardation 1
FMR2	fragile X mental retardation 2
GEF	guanine nucleotide exchange factor
HGP	human genome project
IQ	intelligence quotient
Mb	megabase
MECP2	methyl-CpG-binding protein 2
MIM	mendelian inheritance in man
MR	mental retardation
MRX	non-syndromic X-linked mental retardation
MRXS	syndromic X-linked mental retardation
OMIM	on-line mendelian inheritance in man
PHD	plant homeodomain
PHF6	PHD finger protein 6
RSK2	ribosomal protein S6 kinase
SD	standard deviation
SOX3	SRY (sex determining region Y)-box 3
XLMR	X-linked mental retardation
XNP	X-linked nuclear protein

CHAPTER 1
Introduction

1.1 Intelligence

The human brain has evolved into an extremely complex organ. It is the largest of all vertebrate brains when compared as a proportion of body mass, and has gained and refined such abilities as conscience, language, thought, reasoning, memory, and the capacity to learn. All of these elements come together to form what we term intelligence. The definition of intelligence from the Encyclopaedia Britannica states that 'intelligence is the ability to adapt effectively to the environment, either by making a change in oneself, or by changing the environment or finding a new one'.

Intelligence, like many physical characteristics, is due to a combination of genetic and environmental factors. The contribution of each of these factors results in a continuum of intelligence levels, from very high to very low, as is also true for physical features, such as height and weight. However, unlike physical features, intelligence is not an object that can be easily measured. The most commonly accepted method for determining a person's intelligence is through intelligence quotient (IQ) tests. These tests, simply, give the quotient of the number of questions answered correctly on an examination style test over the number of questions that a person of that physical age would be expected to answer correctly. Therefore, the mean score is set at 100 (assuming the test is calibrated correctly), and any deviation above or below this score is taken to indicate either higher or lower intelligence, respectively.

Numerous epidemiological studies have found that the distribution of IQ approximates the normal distribution (or Bell) curve (Dingman *et al.*, 1960). That is, 95% of the population are contained within 2 standard deviations (SD; 1 SD = 15)

above or below the mean (IQ = 100). It is the proportion of people for whom IQ falls under 70 (2 SDs below the mean) that are defined as having mental retardation.

1.2 Mental Retardation

1.2.1 Definition of Mental Retardation

Mental retardation (MR) is defined as an IQ of less than 70, concurrent deficits in adaptive functioning (such as daily living skills, social and communication skills), and onset before 18 years (which is considered the upper most limit of cognitive development) (American Psychiatric Association, 1994). MR can be further divided into the following subgroups based on IQ; borderline (70-80), mild (50-69), moderate (35-49), severe (20-34) and profound (<19).

1.2.2 Prevalence of Mental Retardation

The exact prevalence of mental retardation in the population is hard to establish, due largely to ascertainment difficulties at the borderline and mild levels. Studies have found between 0.5% to 3% of the population have an IQ < 70 (Birch *et al.*, 1970; Moser *et al.*, 1983; McLaren *et al.*, 1987; Schaefer *et al.*, 1992; Curry *et al.*, 1997; Roeleveld *et al.*, 1997; Crow *et al.*, 1998). 0.3% to 0.5% of the population are found to be moderately to severely effected (IQ < 50) (Laxova *et al.*, 1977; McLaren *et al.*, 1987; Roeleveld *et al.*, 1997; Chelly, 1999), but the proportion of the population with borderline or mild MR is harder to establish, due to ascertainment difficulties. Individuals with mild MR are more likely to assimilate into the community, or adapt their lives to the extent that their intelligence allows, and hence are never detected as having impaired intelligence.

1.3 Causes of Mental Retardation

Mental retardation encompasses a heterogeneous group of disorders where a great deal of effort has gone into determining its causes, both environmental and genetic, in the general population.

1.3.1 Environmental Causes of Mental Retardation

The majority of environmental causes include pre-, peri- or postnatal events that can result in MR. These include head trauma, infectious diseases (such as rubella, cytomegalovirus and meningitis), premature birth (<37 weeks gestation), perinatal anoxia, or consumption of drugs (such as excess alcohol or cocaine) by the mother whilst pregnant (Chelly *et al.*, 2001; Mendola *et al.*, 2002). These events are estimated to account for 15% of patients with mild MR and 35% of patients with severe MR (Hamel, 1999).

1.3.2 Genetic Causes of Mental Retardation

Despite a thorough medical assessment, in approximately 50% of cases there is no obvious environmental cause for patients with mental retardation (Daily *et al.*, 2000), and therefore these are considered idiopathic, where genetic factors are the likely cause. It is estimated that there may be a genetic cause in 50% of severely mentally retarded patients (Hagberg *et al.*, 1983).

The Online Mendelian Inheritance in Man database (OMIM; www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIM) contains 1063 entries for which "mental retardation" is contained in the phenotypic description. This includes various genetic disorders, including chromosomal abnormalities, autosomal and X-linked disorders.

The most common form of genetically caused severe MR is trisomy 21, or Down Syndrome (MIM 190685), which once occurred in 1 in 650-1000 live births (Hook, 1982), but of which approximately 60% of births are now prevented by prenatal diagnostic approaches (Cheffins *et al.*, 2000). Other chromosomal abnormalities can include trisomies of other chromosomes, or partial trisomies or monosomies (examples include Sutherland *et al.*, 1976; Engelen *et al.*, 2002; McDermid *et al.*, 2002; Riegel *et al.*, 2002, among others). These include interstitial duplications and deletions, which can result in contiguous gene deletion syndromes such as Prader-Willi syndrome (MIM 176270), Angelman syndrome (MIM 105830) and Williams-Beuren syndrome (MIM 194050).

A recent field of research into the genetic causes of MR is the analysis of cryptic subtelomeric rearrangements, and includes deletions, duplications and uniparental disomy. Cytogenetic studies of individuals affected with idiopathic moderate to severe MR and dysmorphic features have found between 3.5% to 10% of cases carry some type of subtelomeric rearrangement (Knight *et al.*, 1999; Slavotinek *et al.*, 1999; Rosenberg *et al.*, 2001; Anderlid *et al.*, 2002; Baker *et al.*, 2002; Rio *et al.*, 2002).

Autosomal genes are undoubtedly involved in MR; however, genes which when mutated result in a consistent non-specific intellectual disability phenotype (non-syndromic mental retardation; see section 1.4.2) have been difficult to identify. Large consanguineous families are required for the mapping and subsequent identification of recessive MR genes. Autosomal dominant disorders require families to be large enough to successfully map the gene, which usually only occurs if the phenotype is quite mild (due to a reduced likelihood of moderately to severely affected males fathering children). Recently one autosomal recessive gene for non-syndromic MR

has been identified, caused by a truncating mutation in a neuronal trypsin gene (Molinari *et al.*, 2002).

Identification of MR genes on the autosomes will be advanced by the current identification of MR genes on the X chromosome. As X-linked MR genes have autosomal family members, or genes that encode proteins that act in the same biological pathways, it is reasonable to postulate there are many autosomal recessive genes which cause MR.

However, for between 25% - 40% of cases with severe MR and most mild MR cases the cause remains unknown, despite thorough clinical evaluation of the affected individual (Daily *et al.*, 2000; Chelly *et al.*, 2001). It is postulated that mutations in genes on the X chromosome are responsible for a large proportion of these individuals, especially males, affected with idiopathic MR.

1.4 X-linked Mental Retardation

X-linked mental retardation (XLMR) is a group of genetic disorders affecting cognitive function where the affected gene is on the X chromosome. Various epidemiological studies have found that males are more frequently affected by mental retardation than females, by a 30% excess (Penrose, 1938; Priest *et al.*, 1961; Lehrke, 1974; Herbst *et al.*, 1980; Partington *et al.*, 2000). From these studies, and in combination with the hemizygous state of the X chromosome, it is now generally accepted that there is an excess of genes for cognitive function on the X chromosome (Lehrke, 1972; Turner *et al.*, 1974; Turner, 1996a; Chelly *et al.*, 2001; discussed further in Chapter 1.4.4). Turner (1996a) proposes that 20-25% of mental retardation found in males is due to mutations in genes on the X chromosome.

1.4.1 Definition and Prevalence

X-linked mental retardation consists of a large phenotypically and genetically heterogeneous group of familial disorders. It is a common medical condition in the population, and has an estimated prevalence of 1.7-1.8/1000 males (Glass, 1991; Turner, 1996b). XLMR disorders are broadly divided into two subgroups; syndromic mental retardation and non-syndromic (or non-specific) mental retardation (Mulley *et al.*, 1992).

This splitting of XLMR families into these two groups was initially instituted to help researchers deal with the large and heterogeneous population that is encompassed by this field. However, as the genes responsible for these disorders began to be identified, the initial description of the disorders often change, and these two groups appear to be merging at the boundaries. An example of this situation is the FRAXA syndrome. FRAXA was initially considered non-syndromic, but once the genetic cause was identified, it became clear that there were in fact consistent clinical and behavioural features associated with the disorder other than the MR. This finding placed FRAXA within the syndromic forms of XLMR. As more genes are being identified, families which were originally classed as non-syndromic due to mild clinical features are now being considered syndromic, a recent example being mutations in *SLC6A8*, which causes XLMR with seizures (Hahn *et al.*, 2002). However, the classification system of syndromic and non-syndromic XLMR is largely still employed in initial diagnosis.

1.4.2 Non-syndromic X-linked Mental Retardation

Non-syndromic XLMR, or MRX, is defined as MR being a non-progressive, genetically heterogeneous condition which affects cognitive function in the absence of other consistent dysmorphic, metabolic or neurologic features (Mulley *et al.*, 1992). The estimated incidence of MRX is 0.9–1.4/1000 males (Herbst *et al.*, 1980; Kerr *et al.*, 1991).

Due to the absence of clinical features to distinguish between this group of MR, the symbol MRX is used for each individual family which satisfies the MRX criteria (Mulley *et al.*, 1992), and is numbered sequentially, beginning with MRX1 (Suthers *et al.*, 1988). To be assigned an MRX number, linkage mapping of the affected family requires a LOD score of +2 between the MR locus and one or more X chromosome markers (Ott, 1985; Mulley *et al.*, 1992).

As of February 2003, there are 79 genetic intervals (many of which overlap) for non-syndromic X-linked mental retardation which have been assigned an MRX number (http://www.gene.ucl.ac.uk/nomenclature/) (Chelly, 2000). Each interval represents a family with MR for which the causative gene has been mapped to the X chromosome. However, there is also a large number of samples from new MRX families held at separate research institutions world wide which have not been published or assigned an MRX number, such that the number of families could easily exceed 500 (J. Gécz, personal communication).

FMR2 was the first gene identified for X-linked non-syndromic mental retardation (Gécz *et al.*, 1996; Gu *et al.*, 1996). MR in these cases is caused by loss of expression of the FMR2 protein, due to expansion of a triplet repeat within the promoter of this gene (Gécz *et al.*, 1996). This is the most common form of non-

syndromic X-linked MR, with a minimum prevalence of 1/50000 males (Brown, 1996).

Recently, positional cloning efforts based on X;autosome translocations, deletion mapping or candidate gene analysis have been greatly advanced due mainly to the progression of the Human Genome Project (HGP; http://www.ncbi.nlm.nih.gov/genome/guide; Lander *et al.*, 2001). The availability of the sequence of the X chromosome (which is rapidly nearing completion) has greatly facilitated the identification of genes located on this chromosome. As evidence of this, there has been a spectacular increase in the number of MRX genes identified, with 14 genes now described in the literature (Table 1). Nine of these genes are found to have mutations exclusively in non-syndromic mental retardation.

Initial estimates of the number of MRX genes suggested a minimum of 8, based on the delineation of the minimum number of non-overlapping intervals (Gedeon *et al.*, 1994). Subsequently, this estimate was revised by Gécz *et al.* in 2000 up to a minimum of 11 genes by considering the extra data available. However, this number has already been surpassed (Table 1). Combined with the fact that not all MRX families which localise to the same interval have mutations within known MRX genes, there can be estimated to be at least 22 MRX genes (by merely doubling the genes per interval), but is likely to be much larger (Gécz *et al.*, 2000).

Despite the increase in the number of MRX genes identified, the majority of genes described thus far each account for less than 1% of the total MRX families. There are however several important genes, in terms of accounting for MRX cases. *FMR2* continues to be the most common causative MRX gene identified in screening. However, mutations in the recently identified *ARX* gene (Strømme *et al.*, 2002b)

Table 1 – Genes for Non-syndromic X-linked Mental Retardation

Gene Name	Year	Gene Function	Reference
Non-syndromic MR Genes			
FMR2	1996	transcriptional coactivator	Gécz *et al.*, 1996; Gu *et al.*, 1996
PAK3	1998	p21-activated kinase	Allen *et al.*, 1998
GDI1	1998	Rab GDP-dissociation inhibitor	Bienvenu *et al.*, 1998; D'Adamo *et al.*, 1998
OPHN1	1998	Rho-GTPase activating protein	Billuart *et al.*, 1998
IL1RAPL1	1999	transmembrane, protein signalling	Carrie *et al.*, 1999
TM4SF2	2000	transmembrane, protein signalling	Zemni *et al.*, 2000
ARHGEF6	2000	GEF for Rho-GTPases	Kutsche *et al.*, 2000
FACL4	2002	fatty acid-CoA ligase	Meloni *et al.*, 2002
AGTR2	2002	angiotensin receptor	Vervoort *et al.*, 2002
Syndromic MR Genes involved in Non-syndromic MR			
RSK2	1999	growth factor induced kinase	Merienne *et al.*, 1999
MECP2	2000	methyl-CpG binding protein	Orrico *et al.*, 2000; Couvert *et al.*, 2001
XNP	2002	chromatin remodelling protein	Yntema *et al.*, 2002
FGD1	2002	homology to Ras-like GEFs	Lebel *et al.*, 2002
ARX	2002	Aristaless-related homeobox	Bienvenu *et al.*, 2002; Strømme *et al.*, 2002a

seem to be responsible for the majority of MRX families mapping to Xp22.1. Further studies have found that *ARX* is a very important gene in MRX (Bienvenu *et al.*, 2002; Strømme *et al.*, 2002a). *AGTR2* is also an important MRX gene, with mutations identified in 1.3% of sporadic MR cases screened (Vervoort *et al.*, 2002).

It is interesting that a large proportion of MRX genes appear to play a role in intercellular signalling pathways, specifically within the MAPK / Rho GTPase signalling cascades (reviewed in Chelly, 2000; Gécz *et al.*, 2000). This information on the function of known MRX genes is valuable, to a certain extent, in assessing the candidacy of new MRX genes, particularly when analysing large linkage regions.

1.4.3 Syndromic X-linked Mental Retardation

This group of XLMR (MRXS) is composed of X-linked syndromes of which MR is only one phenotypic feature of an affected individual, in that there are other physical abnormalities and/or biochemical markers associated with the syndrome (Mulley *et al.*, 1992). There are no recent studies on the prevalence of all syndromic XLMRs in the population, as they are not often grouped together as in the manner of non-syndromic forms. A rudimentary calculation (based on total XLMR prevalence = MRX prevalence + MRXS prevalence) gives MRXS an incidence of 0.3-0.9/1000 males.

Syndromic XLMR is named based on either the most distinctive clinical feature associated with the disorder, or eponomously after the dysmorphologists whom described the clinical features of the syndrome (Gécz *et al.*, 2000). The advantage of gene identification for syndromic XLMRs is that mapping information from separate families can be pooled, provided the clinical diagnosis is accurate and there is no genetic heterogeneity (Chelly, 1999).

The most important syndromic XLMR gene, *FMR1*, was identified in 1991 (Oberle *et al.*, 1991; Verkerk *et al.*, 1991; Yu *et al.*, 1991), and accounts for 15 – 20% of syndromic XLMR cases (Turner *et al.*, 1996). The number of identified MRXS genes has also increased in the last decade, again largely attributable to the availability of the human genome sequence information. From combining the latest XLMR update (Chiurazzi *et al.*, 2001) and literature searches to cover the intervening 2 years, there are currently 140 syndromic XLMRs, of which 133 are recessive and 7 are dominant. Of these, the gene responsible has been identified for 35 of these syndromes (Table 2).

Different, or even the same, mutations in one gene can manifest as a range of clinical phenotypes. There are several cases of clinically distinct syndromes due to mutations in the same gene. An example of this is the 7 clinically distinct syndromes caused by mutations in the *XNP* gene: ATR-X, Juberg-Marsidi, Holmes-Gang, Carpenter-Waziri, Smith-Fineman-Myers, Sutherland-Haan, and a syndrome described by Martinez *et al.* (1998) (reviewed in Hamel *et al.*, 2000). In addition, there are also examples of the same gene being involved in both syndromic and non-syndromic forms of XLMRs. This was originally demonstrated in the gene *RSK2*, in which mutations can result in either Coffin-Lowry syndrome or non-syndromic MR (Trivier *et al.*, 1996; Merienne *et al.*, 1999). Since this time, four other genes have also been found which can cause both syndromic and non-syndromic MR; *MECP2* (Orrico *et al.*, 2000; Couvert *et al.*, 2001); *XNP*, (Yntema *et al.*, 2002); *FGD1*, (Lebel *et al.*, 2002); and *ARX* (Bienvenu *et al.*, 2002; Strømme *et al.*, 2002a). *ARX* is an important gene in which different mutations, or even the same mutations, can result in a variety

Table 2 – Genes for Syndromic X-linked Mental Retardation

Malformation XLMR Syndromes

MIM No.	Syndrome Name	Gene	Function	Reference
305400	Aarskog-Scott	*FGDY*	Rho/rac GEF	Pasteris *et al.*, 1994
301040	ATR-X	*XNP*	chromatin remodelling	Gibbons *et al.*, 1995
301900	Börjeson-Forssman-Lehmann	*PHF6*	nucleolin protein	Lower *et al.*, 2002
303600	Coffin-Lowry	*RSK2*	ribosomal protein kinase	Trivier *et al.*, 1996
305000	Dyskeratosis congenita	*DKC1*	ribosomal RNA processing protein	Heiss *et al.*, 1998
309550	Fragile X	*FMR1*	ribosomal RNA binding protein	Yu *et al.*, 1991
300000	Opitz G/BBB	*MID1*	microtubule anchoring protein	Quaderi *et al.*, 1997
309510	Partington	*ARX*	homeobox protein	Strømme *et al.*, 2002b
300055	PPM-X	*MECP2*	methyl-CpG binding protein	Klauck *et al.*, 2002
312870	Simpson-Golabi-Behmel	*GPC3*	cell surface proteoglycan	Pilia *et al.*, 1996
309580	Smith-Fineman-Myers	*XNP*	chromatin remodelling	Villard *et al.*, 2000
309470	Sutherland-Haan	*XNP*	chromatin remodelling	Lossi *et al.*, 1999

Neuromuscular XLMR Syndromes

MIM No.	Syndrome Name	Gene	Function	Reference
310200	Duchenne muscular dystrophy	*DMD*	muscle strength protein	Hoffman *et al.*, 1987
312920	SPG2	*PLP*	myelin component	Saugier-Veber *et al.*, 1994
307000	HSAS	*LICAM*	cell adhesion molecule	Rosenthal *et al.*, 1992
304700	Mohr-Tranebjaerg	*TIMM8A*	mitochondrial import protein	Jin *et al.*, 1996

MIM No.	Syndrome Name	Gene	Function	Reference
310600	Norrie	*NDP*	growth factor	Chen *et al.*, 1992
312080	Pelizaeus-Merzbacher	*PLP*	myelin component	Hudson *et al.*, 1989
300067	SCLH/XLIS	*DCX*	microtubule development	Gleeson *et al.*, 1998
308350	West	*ARX*	homeobox protein	Strømme *et al.*, 2002b
300036	XLMR with seizures	*SLC6A8*	creatine transporter	Hahn *et al.*, 2002

Metabolic XLMR Syndromes

MIM No.	Syndrome Name	Gene	Function	Reference
300100	adrenoleukodystrophy	*ALD*	ATP-binding cassette transporter	Mosser *et al.*, 1993
307030	hyperglycerolemia	*GK1*	glycerol kinase	Walker *et al.*, 1993
309900	Hunter disease	*IDS*	sulfatase	Wilson *et al.*, 1990
300322	Lesch-Nyhan	*HPRT1*	phosphoribosyltransferase	Gibbs *et al.*, 1987
309000	Lowe	*OCRL1*	phosphatase	Attree *et al.*, 1992
309850	MAO-A deficiency	*MAOA*	monoamine oxidase	Brunner *et al.*, 1993
309400	Menkes	*ATP7A*	copper transporting ATPase	Chelly *et al.*, 1993
311250	OTC deficiency	*OTC*	ornithine transcarbamylase	Rozen *et al.*, 1985
311800	PGK1 deficiency	*PGK1*	glycolysis enzyme	Michelson *et al.*, 1983
312170	Pyruvate DH deficiency	*PDHA1*	pyruvate dehydrogenase	Endo *et al.*, 1989

Dominant XLMR Syndromes

MIM No.	Syndrome Name	Gene	Function	Reference
300049	BPNH	*FLN1*	actin-crosslinking phosphoprotein	Fox *et al.*, 1998
308300	Incontentia pigmenti	*NEMO*	activates transcription factor	Smahi *et al.*, 2000
311200	OFD1	*CXORF5*	coiled-coil alpha-helical domain protein	Ferrante *et al.*, 2001
312750	Rett	*MECP2*	methyl-CpG binding protein	Amir *et al.*, 1999

of syndromic and non-syndromic MRs (Bienvenu *et al.*, 2002; Kitamura *et al.*, 2002; Strømme *et al.*, 2002a; Strømme *et al.*, 2002b). The variety of phenotypes which can arise from the same mutation in *ARX*, both intra- and inter-familial, is interesting, and may be partially explained by the varying genetic background among patients.

As to any commonality in the function of the genes involved in syndromic XLMRs, many of the genes appear to play a role in early developmental processes, or are upstream regulators of several other genes (Table 2). It is this loss of 'global' function which most likely results in the pleiotropic effects and complex phenotype observed in many of these syndromes (reviewed in Chelly, 1999). For this reason, syndromic MRs represent a major challenge to the possibility of effective gene therapy ever being applicable.

It is also this complex phenotype which makes it difficult to show a direct link between genes involved in syndromic mental retardation and the role they may play in cognition. This is because the genetic effect of the mutated gene generally acts on various biological pathways, and often it is not clear whether the cognitive phenotype is due directly to the mutated protein or indirectly due to other pathways being disrupted. For example, the causative gene may not even be expressed in the brain, but the observed mental retardation may result from the physically constraining effects of microcephaly. Therefore, the identification of genes involved in syndromic mental retardation disorders usually sheds more light on developmental pathways than on cognitive function.

1.4.4 Excess of Genes for Cognitive Function on the X: Fact or Fiction?

There are many studies in the scientific literature on the hypothesis of there being an excess of genes involved in cognitive function on the X chromosome. Whilst the

higher variability of IQ in males is not disputed, the genetic cause of this finding is often discussed.

Historically, the common explanation for mental retardation in males was due to "the greater size of the male head, exposing the infant to greater difficulties and injuries during labour" (Ireland, 1900). Later, other explanations have been offered, such as ascertainment bias due to higher social expectations placed on boys compared to girls (Penrose, 1938; Nance *et al.*, 1972). A study by Mann *et al.* (1990), wherein the same battery of tests were administered to girls and boys in both Japan and America, found that sex-related differences in cognitive abilities are independent of culture, at least in this instance. In addition, social expectations do not appear to be the cause of this imbalance as this difference in ratio is still being identified in studies being conducted into the 21st century (Partington *et al.*, 2000), when differences in social expectation between the sexes are arguably non-existent.

It has also been suggested that a simpler explanation would be the variable effect of testosterone on males, which may affect cognitive function. The results from this field of work are far from convincing, with different studies finding quite variable results. Examples include a study by Christiansen *et al.* (1987) which found that testosterone had a significantly positive correlation with measures of spatial ability and field dependence-independence, and a significantly negative correlation with measures of verbal production in men only. Tan *et al.* (1992) conversely found an overall positive correlation between fluid intelligence levels and testosterone in both men and women. Whilst these studies have in some cases shown correlations, a definitive causative role of testosterone on intelligence is yet to be offered.

The most commonly touted explanation for the perceived excess of genes for cognitive ability on the X chromosome is simply that there is greater ease in

recognising X-linked forms of MR over autosomal recessive MR. Of the 1063 entries in OMIM with mental retardation, 170 are found on the X chromosome. This represents 16.0% of all mental retardation entries mapping to the X chromosome, four times the expected 4.0% (if shared equally among the 23 autosomes, X and Y chromosomes).

An elegant *in silico* study by Zechner *et al.* (2001) attempted to remove any X chromosome bias by searching OMIM for mental retardation as well as other phenotypic key words, such as polydactyly and cleft palate, and comparing the X-linked entries to the autosomal entries for all features. This study found that after compensating for the ease of identifying genes on the X for any feature, mental retardation is still 3.1 times more frequently associated with X-chromosomal genes than with autosomes (Zechner *et al.*, 2001).

Clearly, the next part to this question and arguably the more important part, once the statistics of the differences between the sexes is laid to rest, is the biological question of why there would be an excess of genes involved in cognitive function on the X chromosome. Recent theories have gone some way to answering this question, by linking intelligence with sexual selection (Zechner *et al.*, 2001), but it appears that as more XLMR genes are identified, they largely give rise to more questions than answers.

1.5 Börjeson-Forssman-Lehmann Syndrome

1.5.1 Original Description

Börjeson-Forssman-Lehmann syndrome (BFLS) was first described in 1962 (Börjeson *et al.*, 1962). The opening sentence of the initial publication reads "We have encountered three extremely feebleminded men, all of a peculiarly grotesque

appearance" (Börjeson *et al.*, 1962), which gives an indication of the severity of the physical and intellectual abnormalities associated with this disorder. The original family consisted of 3 affected male relatives, two maternal half-brothers and a maternal half-uncle. The phenotype of these three individuals consisted of grave mental deficiency with IQ's between 20 and 30, hypogonadism, obesity, grotesque face with fatty swelling of the soft tissues and large ears. All three were short in stature, and two suffered from epilepsy (Börjeson *et al.*, 1962).

1.5.2 Prevalence of Börjeson-Forssman-Lehmann Syndrome

Since the initial description of this syndrome, there have been 6 familial and several sporadic BFLS cases published (Börjeson *et al.*, 1962; Baar *et al.*, 1965; Weber *et al.*, 1978; Robinson *et al.*, 1983; Ardinger *et al.*, 1984; Flannery *et al.*, 1985; Dereymaeker *et al.*, 1986; Mathews *et al.*, 1989; Turner *et al.*, 1989; Petridou *et al.*, 1997; Kubota *et al.*, 1999; Kaplinsky *et al.*, 2001).

The apparent rarity of this disorder may be due to several reasons. Firstly, it is possible that severe mutations in the BFLS gene may be lethal, and hence only a small subset of embryos with mutations in this disease gene may be carried to full term and survive early childhood. Difficulties in ascertainment may also cause these low numbers, as BFLS is not a well known disorder and the differential diagnosis can include Wilson-Turner, Prader-Willi, Klinefelter and Cohen syndromes (Turner *et al.*, in preparation). There are also at least 3 other syndromes for which the gene localises to the same region of the X chromosome as the BFLS gene (Gustavson *et al.*, 1993; Christianson *et al.*, 1999; Shashi *et al.*, 2000), but are currently classed as discrete disorders based on clinical features. As to whether these disorders could be allelic, as

is the case with the number of syndromes which are caused by mutations in the *XNP* gene (as discussed in Chapter 1.4.3), is addressed in this study (see Chapter 2.2).

In addition, there is also one family with non-syndromic mental retardation which has been localised to an overlapping region on chromosome X, MRX27 (Gedeon *et al.*, 1996a). As previously mentioned, it has been shown that different mutations in the same gene can be responsible for both syndromic and non-syndromic forms of mental retardation, and therefore the possibility that this family is allelic with BFLS is also addressed in this study (see Chapter 2.2).

1.5.3 Clinical Description in Affected Males

Since the initial description, there have been a number of papers published documenting both familial and sporadic cases of BFLS, such that the clinical picture of this disorder continued to become clearer over the next four decades. The clinical descriptions from these publications have been combined in Table 3, and present an interesting picture of the common form of BFLS.

There are notably some features which are found in all affected BFLS males, including some level of mental deficiency, although the level of impairment can vary from IQ's of 20 (Börjeson *et al.*, 1962) through to 60 (Robinson *et al.*, 1983). Hypotonia as infants is also a very consistent feature. Underdeveloped sexual organs is another highly consistent feature, with 100% of affected males studied for these characteristics having a small penis, small and/or undescended testes, and delayed secondary sexual characteristics.

Two other features which are highly consistent, and are potentially the most important in terms of a diagnosis of BFLS, are the presence of large but not deformed

Table 3 – Clinical features of BFLS in Affected Males and Carrier Females

Characteristic	Males	Females
Growth and Function		
Postnatal growth deficiency	18/19	6/10 +[1]
Obesity	12/16	5/7 +
Mental deficiency	19/19	7/12 +
Hypotonia	12/12	3/4 +
Craniofacies		
Microcephaly	9/13	3/6
Coarse facial appearance	13/15	6/9 +
Prominent supraorbital ridge & deep set eyes	13/14	5/9 +
Large ears	19/19	6/9 +
Eyes		
Ptosis	12/12	1/8 +
Nystagmus	5/6	1/5
Poor vision with eye abnormalities	10/13	3/10
Genitalia		
Small penis	17/17	-
Small and/or undescended testes	20/20	-
Delayed secondary sex. characteristics	15/15	5/10
Skeletal		
Short stature	4/6	2/2 +
Small hands with tapering fingers	7/9	8/8 +
Variable radiographic abnormalities	10/11	3/5
Other		
Epilepsy	4/8	2/2 +
Gynecomastia	8/8	-

Summary of clinical descriptions taken from Börjeson *et al.*, 1962; Baar *et al.*, 1965; Weber *et al.*, 1978; Ardinger *et al.*, 1984; Flannery *et al.*, 1985; Dereymaeker *et al.*, 1986; Mathews *et al.*, 1989; Turner *et al.*, 1989; Petridou *et al.*, 1997; Kubota *et al.*, 1999; and Kaplinsky *et al.*, 2001.

[1] Female with 100% skewed X-inactivation and showing full BFLS phenotype.

ears (particularly long in the ear lobes), and gynecomastia, or the development of breast tissue (Table 3).

Other features found in the primary BFLS family described, such as short stature and epilepsy, have clearly not been seen in a large number of other affected BFLS males, suggesting that possibly these features are present in only a small number of patients. Comparison of the subsequent BFLS publications does suggest that the original BFLS family was quite severe, and has until now given a somewhat distorted view of the correct clinical picture of BFLS.

The majority of BFLS affected males identified in the last four decades have either a milder version of the features seen in this original family, or are lacking some features all together. This then begs the question as to whether or not this original family in fact has the same syndrome that families subsequently diagnosed with BFLS have (and have since been found to carry *PHF6* mutations; see Chapter 2.2). This question highlights the importance of obtaining samples from the original BFLS family in order to screen for *PHF6* mutations.

1.5.4 Clinical Description in Carrier Females

Female carriers of this disorder have been documented to have variable expressivity of some features of this syndrome, from phenotypically normal to developmentally delayed with some physical dysmorphisms (Turner *et al.*, in preparation). In one large family, obligate female carriers were noted to display the shortened "hammer" toes phenotype as seen in affected males, and it was suggested this feature could possibly be used as an indicator of carrier status (Gedeon *et al.*, 1996b).

One publication describes a female affected with the severe form of BFLS, similar to affected males (Kubota *et al.*, 1999). Analysis of her X inactivation status in blood

lymphocytes found that her X chromosomes were completely skewed, such that the same X chromosome was active in all her cells. There is no indication of what caused the skewing of X inactivation in this female, as her karyotype analysis was normal, however a defect in a gene or genes involved in the X inactivation process is likely. This would therefore explain her phenotype if the gene carrying the BFLS mutation was contained on the active X, in essence giving her the gene expression profile of a male affected with BFLS. Therefore, this individual was not included in the phenotypic classification table for carrier females, so as not to unduly skew the results. Her features are represented separately in Table 3 as + for the presence of a specific feature. Again, this individual needs to be examined for a *PHF6* mutation to confirm this hypothesis.

The most obvious point to make for BFLS carrier females is the variability of the presence of features, with the only consistent feature being small hands and tapering fingers observed in all carriers. Clearly, this disorder is not fully penetrant in females, potentially due to the variability of skewed X-inactivation (Lower *et al.*, 2002).

1.5.5 Genetic Mapping of the BFLS Disease Gene

BFLS was first speculated to be an X-linked disorder when it was initially described by considering the inheritance pattern seen in the affected family (Börjeson *et al.*, 1962). However, it was not until molecular biology techniques rapidly expanded 2 decades later that this mode of inheritance could be confirmed experimentally. The localisation of the disease gene on the X chromosome was initially mapped to a large region on the long arm of the X chromosome (Mathews *et al.*, 1989; Turner *et al.*, 1989). With the increasing number of polymorphic markers, due to the progress of

the Human Genome Project, and an additional 11 members to a previously described large BFLS family, Gedeon *et al.* (1996b) reduced this localisation to Xq26 – Xq27, between the markers *DXS425* and *DXS105*. This was a region of 24.6 cM based on the genetic map used (Gedeon *et al.*, 1996b), which can roughly translate to 25 Mb of genomic DNA. Based on current estimates provided by the Human Genome Project sequence information (http://www.ncbi.nlm.nih.gov/genome/guide/human), this size of region on the X chromosome could contain at least 200 genes.

1.5.6 Candidate Genes Analysed

Due to this potentially large number of genes, the candidate gene approach had not previously been actively undertaken, with only 2 genes screened for mutations in BFLS individuals. The HMG-box domain of the *SOX3* gene has been analysed and no mutations were found in BFLS patients (Gedeon *et al.*, 1996b). The gene for fibroblast growth factor homologous factor 2 (*FHF2*) is interrupted by a duplication breakpoint in a BFLS-like patient (Gécz *et al.*, 1999), but mutations in *FHF2* were not found in familial cases mapping to the same region (Gécz, unpublished).

1.6 Research Aims

The aims of my PhD research were as follows :

1) To reduce the region of the BFLS gene using various methods, in an attempt to reduce the localisation to a practical region for candidate gene analysis;
2) To obtain genetic material from other BFLS families from overseas through scientific collaborations;
3) To develop a detailed physical and transcript map of the region to which the BFLS gene localises;
4) To weight the candidacy of genes which are contained within the BFLS localisation;
5) To undertake mutation detection of these candidate genes within BFLS affected individuals; and ultimately
6) To detect molecular defects linking the putative gene to the disease; and
7) Once identified, to undertake preliminary analysis of the gene's function based on expression patterns and cellular localisation of the protein.

The thesis describes, in the form of 3 papers published in scientific journals, the extent to which the above aims have been achieved during the course of my PhD candidature. In addition, 3 further publications are included as supplementary research, and involve the identification of another mental retardation gene, *ARX*, and two manuscripts (one published and one in preparation) which are a direct result from my work to identify the gene responsible for Börjeson-Forssman-Lehmann syndrome.

CHAPTER 2

Thesis Publications

2.1 Publication 1

2.1.1 Linking Statement

As the aim of the project was to identify the gene responsible for BFLS, this entailed mutation analysis of a number of genes identified within the BFLS critical region. One of the genes which I screened was *ARHGEF6*, a guanine nucleotide exchange factor for Rho GTPases.

This gene was an excellent candidate for BFLS, for several reasons. Firstly, it mapped within the identified candidate gene region for BFLS. Secondly, the tissue-specific expression pattern of *ARHGEF6*, which included the brain, correlated well with the phenotype of BFLS patients. However, the strongest reason I selected this gene as a candidate for BFLS was due to its interactions with p21-activated kinases (PAKs), one of which, *PAK3*, is a known MRX gene.

The following publication details the placement of *ARHGEF6* within the BFLS candidate region, delineation of the full coding sequence and genomic structure of the gene, analysis of expression patterns of the gene, and mutation screening which I undertook in BFLS and MRX27 patient DNA.

Whilst this gene did not cause BFLS or MRX27, this publication was very timely due to the fact that as this paper was being reviewed, Kutsche *et al.* (2000) reported two cases of non-specific mental retardation where this gene is disrupted. Although *ARHGEF6* was not responsible for BFLS, the finding by Kutsche *et al.* (2000) reinforced the selection criteria used in the BFLS project for assessing potential candidate genes within the BFLS critical region. In addition, the information contained within this publication, including the complete coding sequence of the

gene, the exon/intron structure, and the primer sequences used for amplification of these products, is now available for other groups to examine this gene as a candidate for other disorders that map to the same region of the X chromosome.

Lower, K,M., and Gecz. J., (2001) Characterization of ***ARHGEF6***, a guanine nucleotide exchange factor for Rho GTPases and a candidate gene for X-linked mental retardation: Mutation screening in Börjeson-Forssman-Lehmann syndrome and MRX27.
American Journal of Medical Genetics, v. 100 (1), pp. 43-48.

2.2 Publication 2

2.2.1 Linking Statement

The following publication in Nature Genetics reports the most significant outcome of my PhD. The aim of my PhD research was to identify the gene responsible for BFLS, and this paper describes that finding, albeit in the compact format required by Nature Genetics.

The publication outlines those initiatives which reduced the BFLS candidate region to a smaller critical interval, and the identification of at least 62 potential candidate genes within this interval. I identified mutations in a novel PHD-like zinc finger gene, which has been named *PHF6*. This paper details the spectrum of mutations that I initially identified from 9 familial and sporadic cases of BFLS within this gene.

In addition, the paper contains characterisation of *PHF6*, including its exon/intron structure and its expression pattern in human tissues and mouse brain. I also analysed the protein product, PHF6, in terms of the full amino acid sequence, identification of orthologues in various vertebrate species, and subcellular localisation using green fluorescent protein expression constructs. In particular, the functionality of the nuclear localisation sequences was analysed, in order to understand the nucleolar localisation of the protein.

This publication encapsulates the bulk of 3 years of my PhD research, and in essence represents the achievement of all of my research goals, as laid out at the beginning of my candidature for my PhD.

Lower, K.M., Turner, G., Kerr, B.A., et al., (2002) Mutations in PHF6 are associated with Börjeson-Forssman-Lehmann syndrome.
Nature Genetics, v. 32 (4), pp. 661-665.

Web table A

Unigene Cluster	Genbank Acc. No.	Characterised Gene/ EST Cluster	LocusLink	OMIM	Expression in brain
152292	NM_003069	*SMARCA1**	6594	300012	Yes
181060	NM_017413	*Apelin**	8862	300297	Yes
57922	NM_003399	*XNPEP2*	7512	300145	No
248476	AI762422	EST cluster	-	-	No
181060	NM_001587	*OCRL*	4952	309000	Yes
126357	AI733007	EST cluster	-	-	No
61469	NM_018990	*753P9**	54440	-	Yes
63970	N21373	EST cluster	-	-	Yes
271926	NM_006649	*SDCCAG16**	10813	-	Yes
8929	AK021424	hypothetical protein*	63035	-	Yes
151139	NM_001421	*ELF4**	2000	-	Yes
18720	AF100928	*PDCD8*	9131	300169	Yes
56294	NM_004794	*RAB33A**	9363	-	Yes
230873	AI494423	EST cluster	-	-	Yes
121668	AI923562	EST cluster	-	-	No
194686	NM_022810	*SLC25A14**	9016	300242	Yes
130231	AI090665	EST cluster	-	-	Yes
61184	NM_016024	*CGI79**	51634	-	Yes
267923	AK027029	*HTO11**	55855	-	Yes
155185	NM_006375	*COVA1**	10495	300282	Yes
22111	NM_001555	*IGSF1*	3547	300137	Yes

89525	NM_004494	*HDGF**	3068	300043	Yes
23643	NM_016542	*MST4**	51765	-	Yes
170776	AL161984	hypothetical protein	-	-	No
225979	AL049685	hypothetical protein*	57826	-	Yes
35225	NM_018388	hypothetical protein	55796	-	No
282157	AI913335	EST cluster	-	-	No
146213	AI276039	EST cluster	-	-	No
82302	AA633866	EST cluster	-	-	No
142908	NM_016521	*E2F-like*	51270	-	No
58367	NM_001448	*GPC4*	2239	300168	Yes
258966	AW590088	EST cluster	-	-	No
269863	R07953	EST cluster	-	-	No
125958	AI638470	EST cluster	-	-	No
189745	T90927	EST cluster	-	-	No
269126	N68607	EST cluster	-	-	No
34081	R86779	EST cluster	-	-	No
119651	NM_004484	*GPC3*	2719	300037	Yes
213766	AI916456	EST cluster	-	-	No
356501	AI474638	EST cluster	84295	-	Yes
82314	NM_000194	*HPRT1*	3251	308000	Yes
108785	AI334376	EST cluster	-	-	Yes
268566	T24013	EST cluster	-	-	No
13026	NM_021796	*PLAC1*	10761	300296	No
5206	AA243478	EST cluster	-	-	No
288550	AI969411	hypothetical protein	-	-	Yes

46908	AI629041	EST cluster	-	-	Yes
131422	AI023601	EST cluster	-	-	No
192806	AI610994	EST cluster	-	-	No
42982	AI653705	EST cluster	-	-	No
114689	R93330	EST cluster	-	-	No
57549	NM_019556	hypothetical protein*	56180	-	Yes
284266	NM_032628	hypothetical protein	26071	-	No
190379	AI248159	EST cluster	-	-	No
42239	AI252679	EST cluster	-	-	No
19114	NM_005342	*HMG4**	3149	300193	Yes
250708	AF038168	*CXX1**	8933	300213	Yes
239069	U60115	*FHL1**	2273	300163	Yes
62185	NM_006359	*SLC9A6*	10479	300231	Yes
121484	L08893	*BRS3*	680	300107	Yes
171595	U76992	*TAT-SF1*	27336	300346	Yes
9030	AF137387	*TONDU*	51442	-	No

The 18 genes/EST clusters screened negative in the BFLS cases tested are indicated with an asterisk (*). The *PHF6* gene cluster is underlined.

2.3 Publication 3

2.3.1 Linking Statement

The following publication is a review on X-linked mental retardation, which I wrote in collaboration with my supervisor, Jozef Gécz, and a fellow PhD student, Marie Mangelsdorf. The aim of this review was to discuss the field of X-linked mental retardation from the aspect of the scientific knowledge available at that time, the direction that the field was progressing at that time, and where we thought the field may be in 5 or 10 years time, including the application of potential therapies.

This review also highlighted the resources available for research into this area.

Lower, K., Mangelsdorf, M., and Gecz, J., (2001) Molecular genetics of X-linked mental retardation: a complex picture emerging.
Expert Review of Molecular Diagnostics, v. 1 (2), pp. 220-225.

CHAPTER 3

Publications Supplementary to Thesis

3.1 Publication 4

3.1.1 Linking Statement

The following publication documents a novel *PHF6* mutation in a large BFLS family. The mutation is of particular interest as it is a 3 base pair deletion mutation, which removes a single amino acid at the carboxyl terminal end of the PHF6 protein. Prior to this finding, all other *PHF6* mutations identified were single base pair changes that altered a single amino acid or resulted in a truncated protein (Lower *et al.*, 2002). Of further interest is the fact that carrier females within this family do not display skewed X-inactivation in their blood leucocyte DNA, a feature that we have found consistently in other BFLS families where *PHF6* mutations have been identified.

My role in this work was to supply our collaborators in Germany with the sequence of the *PHF6* gene, and the conditions required to screen the gene for mutations. Following the identification of the above mutation in their BFLS family, I was also involved in critical reading of the manuscript.

This publication is submitted as part of this thesis as work that has arisen as a direct result of my identification of the gene responsible for this disorder.

Baumstark, A., Lower, K.M., Sinkus, A., Andriuškevičiūtė, I., Jurkėnienė, L., Gecz, J., and Just, W., (2003) Novel *PHF6* mutation p.D333del causes Börjeson-Forssman-Lehmann syndrome.
Journal of Medical Genetics, v. 40 (4), pp. e50.

3.2 Publication 5

3.2.1 Linking Statement

The following publication, which is in preparation for submission to Clinical Genetics, describes for the first time a clear clinical picture of BFLS in *PHF6* mutation-positive individuals. The findings include that the BFLS phenotype is milder than first described, and also that clinical features change with age. It also discusses the female phenotype in detail for the first time, with reference to skewed X-inactivation and clinical features. This publication speculates that BFLS may be under diagnosed in males, and not considered in carrier females who are affected.

Again, this publication is included as supplementary to the work described within this thesis, as it is a direct result of the identification of *PHF6* mutations causing this disorder (Lower *et al.*, 2002).

Turner, G., Lower, K.M., White, S.M., et al., (2004) The clinical picture of the Börjeson-Forssman-Lehmann syndrome in males and heterozygous females with PHF6 mutations.
Clinical Genetics, v. 65 (3), pp. 226-232.

3.3 Publication 6

3.3.1 Linking Statement

The laboratory in which I undertook my PhD research, led by Jozef Gécz, is specifically focussed on the identification of genes involved in X-linked mental retardation, hence my research into the genetic cause of BFLS. During my candidature, a research group of Marie Mangelsdorf, Peter Strømme, and Jozef Gécz made an outstanding discovery of another gene involved in X-linked mental retardation, *ARX*.

ARX is a novel aristaless-related paired-class homeodomain gene which we originally identified in our laboratory as responsible for various XLMR syndromes, including West syndrome, ISSX and Partington syndrome (Strømme *et al.*, 2002b). The following publication arose from a team effort, in which I played a role in haplotype analysis and mutation detection.

Research on this gene has since gone on to show its involvement in a number of other X-linked mental retardation families, both syndromic and non-syndromic (Bienvenu *et al.*, 2002; Frints *et al.*, 2002; Kitamura *et al.*, 2002; Strømme *et al.*, 2002a; Turner *et al.*, 2002). This gene quickly became recognised as the second most significant cause of heritable MR, after fragile X syndrome (*FMR1*).

Whilst my role in this work was relatively minor, it relates to the general theme of understanding the molecular basis of mental retardation which is being undertaken in a global sense by the research group.

It is included as part of this thesis as complementary work in the same field as my primary research.

Strømme, P., Mangelsdorf, M.E., Shaw, M.A., et al., (2002) Mutations in the human ortholog of Aristaless cause X-linked mental retardation and epilepsy.
Nature Genetics, v. 30 (4), pp. 441-445.

CHAPTER 4

Conclusions

The six publications contained within this thesis represent the work which I have both generated and contributed to during my PhD studies, with the main focus on the identification and characterisation of the gene responsible for Börjeson-Forssman-Lehmann syndrome (BFLS). The identification of the gene responsible for BFLS is significant for several reasons.

4.1 Historical Significance

BFLS was one of the earliest X-linked mental retardation syndromes described (Börjeson *et al.*, 1962). For four decades the genetic cause of this debilitating disorder was unknown. This research has identified the gene responsible for this disorder, and has gone some way to analysing the function of the resultant protein. Due to a lack of access to patient material from the original BFLS family however, screening of the BFLS gene has not yet been undertaken for affected individuals from this family.

4.2 Significance to Families and Individuals with BFLS

4.2.1. Carrier Female Testing and Pre-natal Diagnosis

The identification of the gene means that for the first time women in families affected with BFLS have access to 100% reliable carrier testing. Prior to this work, females were assessed for their carrier status by linkage, i.e. using polymorphic markers from the BFLS gene region, leading to a probability based assessment of carrier status. However, with the new information gained during the present studies, there is now a

more direct test for carrier status. Females in families with a known mutation now have access to a 100% reliable test for their carrier status. Similarly, if carriers choose to go on and have prenatal diagnosis for future pregnancies, testing for the presence or absence of the mutation known to be present in their family provides a definitive result to the parents. This test is especially useful in families with isolated BFLS cases, where it cannot be determined at which point in the pedigree the mutation occurred, and hence linkage was previously of no use.

The finding that highly skewed X inactivation is present in the majority of BFLS obligate carrier females is a very useful pre-screening tool for diagnosis. As a pre-screening tool, X inactivation testing can be undertaken in familial cases on mothers of presumptive BFLS patients. This analysis gives an indication as to the likelihood of there being a *PHF6* mutation involved. This is especially valuable in terms of resources used on screening sporadic BFLS cases, where fewer are expected to be true BFLS (due to the absence of an accurate diagnostic marker). Unfortunately, it is not helpful for screening purposes in mothers of sporadic males where there is a *de novo* mutation; for instance where the mutation may have originated in the ovum of the mother. Similarly, there is at least one documented BFLS family with *PHF6* mutations where skewed X-inactivation was not found in carrier females (see Chapter 3.1). Therefore, implementing this pre-screening criteria will miss a small proportion of PHF6 mutations. This work is still very preliminary, and further research needs to be conducted to confirm its validity as a diagnostic tool.

4.2.2. Diagnosis of BFLS

The identification of the gene *PHF6* means that clinically diagnosed BFLS individuals can have this diagnosis confirmed by detection of mutations in *PHF6*.

This applies to both individuals in families with a history of BFLS, or in sporadic cases. This is especially useful given the differential diagnosis of BFLS with other mental retardation syndromes, including Wilson-Turner, Prader-Willi, Klinefelter and Cohen syndromes (Turner *et al.*, in preparation). The identification of the genetic cause of BFLS means that individuals who fit with this diagnostic picture can now definitively be included or excluded from having BFLS. This will also give us a clearer insight into the true prevalence of BFLS.

4.3. Significance of Understanding the Function of PHF6

Understanding the function of the PHF6 protein will give important information into how the protein works in normal development and why, when perturbed, the BFLS phenotype is the result.

4.3.1. PHD-like Zinc Fingers

The function of PHF6 is not known, but the presence of the PHD-like domains indicates a role for this protein in protein-protein interactions (Aasland *et al.*, 1995). This motif is found in over 40 eukaryotic proteins, and often occurs in regulatory genes such as members of the trithorax and polycomb (TRX-G and PC-G) genes (Aasland *et al.*, 1995) and leukaemia-associated proteins (Saha *et al.*, 1995). PHD domain-containing proteins have been found to be involved in many human disorders. Most notably, mutations within the PHD domain of the XNP protein result in α-thalassemia and mental retardation (ATR-X syndrome; Gibbons *et al.*, (1995)). Any similarity in function between XNP and PHF6 is yet to be determined.

4.3.2. Nucleolar Localisation of PHF6

Nucleoli are organelles within the nucleus which are assembled around clusters of tandemly repeated ribosomal genes (Dundr *et al.*, 2001). They seem to be predominantly involved in ribosome biogenesis. There is, however, increasing evidence that nucleoli are also involved in many non-ribosomal activities, such as being the site for protein synthesis by the ribonucleoprotein machinery (Pederson *et al.*, 2000), and involvement in the regulation of such proteins as the tumour suppressor *p53* (Prives *et al.*, 1999) and HIV-regulatory proteins (Hope, 1999; Zolotukhin *et al.*, 1999). As to whether PHF6 is involved in one of these functions, or an as yet undescribed nucleolar function is not yet known, and work is currently under way which may help to answer this question.

4.3.3 Orthologues in Other Species

Orthologues of PHF6 were found in various vertebrate species, including mouse, *F. rubripes* (puffer fish), *D. rerio* (zebrafish) and *X. laevis* (frog) (Lower *et al.*, 2002). However, orthologues could not be identified in any non-vertebrate proteomes, including *S. cerevisiae* (yeast) and *C. elegans* (worm). As the genomes for these two organisms have been completely sequenced (Entrez Genome, http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Genome), it appears that no orthologue of PHF6 exists in these species. Whether this implies that PHF6 is a vertebrate-specific protein remains to be seen, as more genomes will have to be sequenced, from both vertebrates and non-vertebrates, before this can be confirmed. However, it is unlikely that vertebrates would not have an orthologue of PHF6, as it is present in the *F. rubripes* proteome, which is considered to be representative of the most primitive vertebrate genome (Aparicio *et al.*, 2002).

4.4 Significance to the Field of X-Linked Mental Retardation

4.4.1 Role of *PHF6* in BFLS

The project has demonstrated that mutations in *PHF6* cause BFLS. However, it is still unclear if all BFLS cases are caused by mutations in *PHF6*. There are BFLS-like cases and cases where the diagnosis is indicative of BFLS, where *PHF6* mutations have not been identified (Lower *et al.*, 2002). There are several possible explanations for this. The mutation detection method that I used (primarily direct sequencing of exonic PCR products amplified from patient DNA) would not have identified mutations such as large intronic deletions, promoter mutations and chromosomal aberrations such as translocations. Similarly, as the clinical diagnosis of BFLS does not have any specific confirmatory physical or biochemical markers, it is also possible the affected individuals were incorrectly diagnosed with BFLS. *PHF6* mutation screening is the first confirmatory tool to become available to clinicians that may be presented with BFLS patients.

4.4.2 Role of *PHF6* in X-linked Mental Retardation

In terms of research into X-linked mental retardation, the identification of the gene for BFLS is another important step in the continuing search for understanding of genetic defects of cognition. It is as yet unclear what proportion of intellectual disability is due to mutations in *PHF6*. To answer this question, a large scale screening of sporadic non-syndromic mental retardation samples would need to be undertaken.

4.5 Final Conclusions

The identification of *PHF6* as the gene responsible for BFLS ends a 40 year search that began with the original description of the syndrome in 1962 (Börjeson *et al.*, 1962). This novel gene is an important regulator of normal development through its effects on many physiological pathways, including those related to cognitive function, sexual development, endocrine function, and craniofacial development. This opens the way to understanding the exact function of PHF6. Only then will we be able to extrapolate to see how this function, when perturbed, results in the phenotype we see in individuals affected with BFLS.

This is not however an easy process. Some proteins involved in intellectual disability syndromes have been studied in great detail, such as FMR1, ATR-X, and MIDI (which is responsible for Opitz G/BBB syndrome). The results thus far, whilst offering an insight into what these proteins might do, do not explain how the absent or altered protein results in the phenotype of these disorders, specifically in intellectual disability. Though ending a 40 year search for the gene, in many ways the discovery of the gene represents only the beginning of the next phase.

As more and more genes are identified which when perturbed affect cognitive function, the next challenge will be to use this information to understand how cognitive function develops and works in the normal physiological system, and the most complex of all human organs, the brain.

4.6 Future Directions

The stage is now set for expansion into new areas for studies of BFLS and *PHF6*.

1) Characterising the mutation in the original BFLS family, for historical reasons;
2) Refine the mutation screening of the BFLS gene, such that it is transferable from the research lab to the diagnostic lab;
3) Screen sporadic non-syndromic MR patients to fully explore the role which mutations in *PHF6* may play in mental retardation in the general population;
4) Yeast 2 Hybrid studies to identify proteins that interact with PHF6, in order to further our understanding of the function of PHF6, and also to identify potential candidates for clinically similar syndromes, such as Wilson-Turner syndrome;
5) Transcription activation and repression studies to ascertain if PHF6 plays a role in regulating transcription of other genes, which may explain the multisystemic effects of PHF6 mutations;
6) Following on from microarray results which have identified genes which appear to be down regulated in BFLS patient cell lines, and thereby extending the pathways affected by PHF6;
7) Generation of an over expression system for PHF6 in a neuronal outgrowth model (rat PC12 cells) to identify morphological changes resulting from over expression of normal and mutant PHF6, in order to identify pathways which are affected by PHF6; and
8) Generation of a PHF6 mouse knockout model to analyse the physiological effect of absence of the BFLS protein on different tissues and cell types.

APPENDICES

Appendix 1

Publication 1 - Author Contributions

Lower, K. M. and Gécz, J. Characterization of *ARHGEF6*, a Guanine Nucleotide Exchange Factor for Rho GTPases and a Candidate Gene for X-linked Mental Retardation: Mutation Screening in Börjeson-Forssman-Lehmann Syndrome and MRX27. 2001. *Am J. Med. Genet.* 100:43-48.

Karen M. Lower – 95% of the experimental work and preparation of the manuscript
Jozef Gécz – supervision of the experimental work and critical reading of the manuscript

I, JOZEF GÉCZ, as a co-author of the following publication,

Lower, K. M. and Gécz, J. Characterization of *ARHGEF6*, a Guanine Nucleotide Exchange Factor for Rho GTPases and a Candidate Gene for X-linked Mental Retardation: Mutation Screening in Börjeson-Forssman-Lehmann Syndrome and MRX27. 2001. *Am J. Med. Genet.* 100:43-48.

agree that Karen Lower carried out 95% of the experimental work contained within said publication.

Date 16/4/2003

Publication 2 – Author Contributions

Lower, K. M., Turner, G., Kerr, B. A., Mathews, K. D., Shaw, M. A., Gedeon, A. K., Schelley, S., Hoyme, H. E., White, S. M., Delatycki, M. B., Lampe, A. K., Clayton-Smith, J., Stewart, H., van Ravenswaay, C. M. A., de Vries, B. B. A., Cox, B., Grompe, M., Ross, S., Thomas, P., Mulley, J. C., and Gécz, J. Mutations in PHF6 are associated with Börjeson-Forssman-Lehmann syndrome. 2002. *Nat. Genet.* 32:661-665.

K. M. Lower – 90% of the experimental work and preparation of the manuscript
G. Turner – contributed family material, gave diagnostic opinion and critical reading of the manuscript
B. A. Kerr – contributed family material
K. D. Mathews – contributed family material
M. A. Shaw – contributed to cellular localisation studies
A. K. Gedeon – undertook original linkage analysis
S. Schelley – contributed family material
H. E. Hoyme – contributed family material
S. M. White – contributed family material
M. B. Delatycki – contributed family material
A. K. Lampe – contributed family material
J. Clayton-Smith – contributed family material
H. Stewart – contributed family material
C. M. A. van Ravenswaay – contributed family material
B. B. A. de Vries – contributed family material
B. Cox – contributed family material
M. Grompe – contributed family material
S. Ross – performed mouse *in situ* studies
P. Thomas – performed mouse *in situ* studies
J. C. Mulley – supervision of project and critical reading of the manuscript
J. Gécz – supervision of project and experimental work, and critical reading of the manuscript

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Lower KM, Turner G, Kerr BA, Mathews KD, Shaw MA, Gedeon AK, Schelley S, Hoyme HE, White SM, Delatycki MB, Lampe AK, Clayton-Smith J, Stewart H, van Ravenswaay CM, de Vries BB, Cox B, Grompe M, Ross S, Thomas P, Mulley JC, Gecz J. Mutations in PHF6 are associated with Börjeson-Forssman-Lehmann syndrome. *Nat Genet* 2002; 32:661-5,

agree that Karen Lower carried out 90% of the experimental work contained within said publication.

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I, _ _____, as a co-author of the following publication,

Lower KM, Turner G, Kerr BA, Mathews KD, Shaw MA, Gedeon AK, Schelley S, Hoyme HE, White SM, Delatycki MB, Lampe AK, Clayton-Smith J, Stewart H, van Ravenswaay CM, de Vries BB, Cox B, Grompe M, Ross S, Thomas P, Mulley JC, Gecz J. Mutations in PHF6 are associated with Borjeson-Forssman-Lehmann syndrome. *Nat Genet* 2002; 32:661-5.

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I, Bronwyn Kerr, as a co-author of the following publication,

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I, Katherine Mathews, as a co-author of the following publication,

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Lower KM, Turner G, Kerr BA, Mathews KD, Shaw MA, Gedeon AK, Schelley S, Hoyme HE, White SM, Delatycki MB, Lampe AK, Clayton-Smith J, Stewart H, van Ravenswaay CM, de Vries BB, Cox B, Grompe M, Ross S, Thomas P, Mulley JC, Gecz J. Mutations in PHF6 are associated with Borjeson-Forssman-Lehmann syndrome. *Nat Genet* 2002; 32:661-5,

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I, HELEN STEWART, as a co-author of the following publication,

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Lower KM, Turner G, Kerr BA, Mathews KD, Shaw MA, Gedeon AK, Schelley S, Hoyme HE, White SM, Delatycki MB, Lampe AK, Clayton-Smith J, Stewart H, van Ravenswaay CM, de Vries BB, Cox B, Grompe M, Ross S, Thomas P, Mulley JC, Gecz J. Mutations in PHF6 are associated with Borjeson-Forssman-Lehmann syndrome. *Nat Genet* 2002; 32:661-5,

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Publication 3 – Author Contributions

Lower, K., Mangelsdorf, M. and Gécz, J. Molecular genetics of X-linked mental retardation: a complex picture emerging. 2001. *Expert Rev. Mol. Diagn.* 1:220-225.

K. Lower – literature searches and manuscript preparation
M. Mangelsdorf – literature searches and manuscript preparation
J. Gécz – literature searches and manuscript preparation

Publication 3 – Author Statements

I, JOZEF GECZ, as a co-author of the following publication,

Lower, K., Mangelsdorf, M. and Gécz, J. Molecular genetics of X-linked mental retardation: a complex picture emerging. 2001. *Expert Rev. Mol. Diagn.* 1:220-225.

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Date 16/4/03

I, MARIE MANGELSDORF, as a co-author of the following publication,

Lower, K., Mangelsdorf, M. and Gecz, J. Molecular genetics of X-linked mental retardation: a complex picture emerging. 2001. *Expert Rev. Mol. Diagn.* 1:220-225.

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