Chapter 1

Introduction and outline

1.1 Periodic catatonia as a complex disease

Schizophrenia is a complex neuropsychiatric disorder of uncertain etiology and pathophysiology, which affects approximately 1% of the population worldwide. Despite intensive study, its molecular etiology remains enigmatic. Like many common diseases, schizophrenia is multifactorial in origin, with both genetic and environmental contributions likely playing an important role in the manifestation of symptoms.

Periodic catatonia (SCZD10, OMIM #605419), a clinical subtype of unsystematic schizophrenias according to Leonhardt's classification, exhibits subtle dearrangements of facial expression and gestures, so-called psychomotor disturbances (Leonhard, 1999). Patients with periodic catatonia express a variable phenotype combining akinetic negativism, hyperkinesias with stereotypies and parakinetic movements as well as increased anxiety, impulsivity, and aggressiveness. In most cases, acute psychotic episodes are accompanied by hallucinations and delusions, but, in remission, there remains a distinct mild to severe catatonic residual state with psychomotor weakness of facial expression. Periodic catatonia patients diagnosed according to Leonhardt´s classification are most probably diagnosed as Bipolar I patients by DSM IV criteria (Taylor and Fink, 2003).

Twin studies unequivocally showed that schizophrenia, although influenced by environmental factors, is predominantly a genetic disorder, with estimates of heritability of risk of around 81% (reviewed in Sullivan *et al*., 2003). Stöber and colleagues (1995) and

Beckmann and co-workers (1996) predicted a major gene effect in periodic catatonia with respect to a morbidity risk of 26.9% in first-degree relatives.

Identification of genetic polymorphisms that contribute to susceptibility for common complex diseases, i.e schizophrenia, will aid in the development of diagnostics and therapeutics. While gene mapping works in breast cancer disease led to the identification of the *BRCA1* and *BRCA2* genes (Strachan & Read, 1999), on the contrary, such attempt in schizophrenia produced scattered susceptibility loci throughout the genome (Levinson *et al*., 2003). However, a result from meta-analysis of schizophrenia genome scan showed a greater consistency of linkage results across studies than has been previously recognized for loci 2q, 5q, 3p, 11q, 6p, 1q, **22q**, 8p, 20q, and 14p, 16q, 18q, 10p, **15q**, 6q, and 17q (Lewis *et al.*, 2003).

Chromosome 15 contains regions shared between different mental disorders. Catatonia is beginning to be seen as a shared syndrome in ASD, SZ and BP, and may be a useful intermediate phenotype (Chagnon, 2006). Therefore, the identification and validation of additional candidate genes on chromosome 15 by fine mapping and association studies should be a priority.

1.2 State of research

In a genome-wide linkage scan, Stöber and colleagues (2000) found evidence for a major susceptibility locus on chromosome 15q15 in most of the pedigrees analyzed (maximum nonparametric LOD score of 3.57), and a further potential locus meeting criteria for suggestive evidence for 22q13, pointing to genetic heterogeneity in periodic catatonia (Figure 1a). In a second genome-wide scan, Stöber et al. (2002) found the overlapping region

with the previous genome-wide scan result on chromosome 15q (Figure 1b) and were able to refine the disease gene locus to an 11-cM region between D15S1042 and D15S659. These genome-wide scan and linkage study are the starting point of present doctoral study for further refinement of catatonic schizophrenia susceptibility loci and analysis of candidate genes. Furthermore, Meyer and colleagues (2001) reported a missense mutation (Leu309Met) in a gene called *MLC1* that was co-segregating with psychosis in the pedigree, which gave signal for chromosome 22q13 in the aforementioned genome-wide scan study.

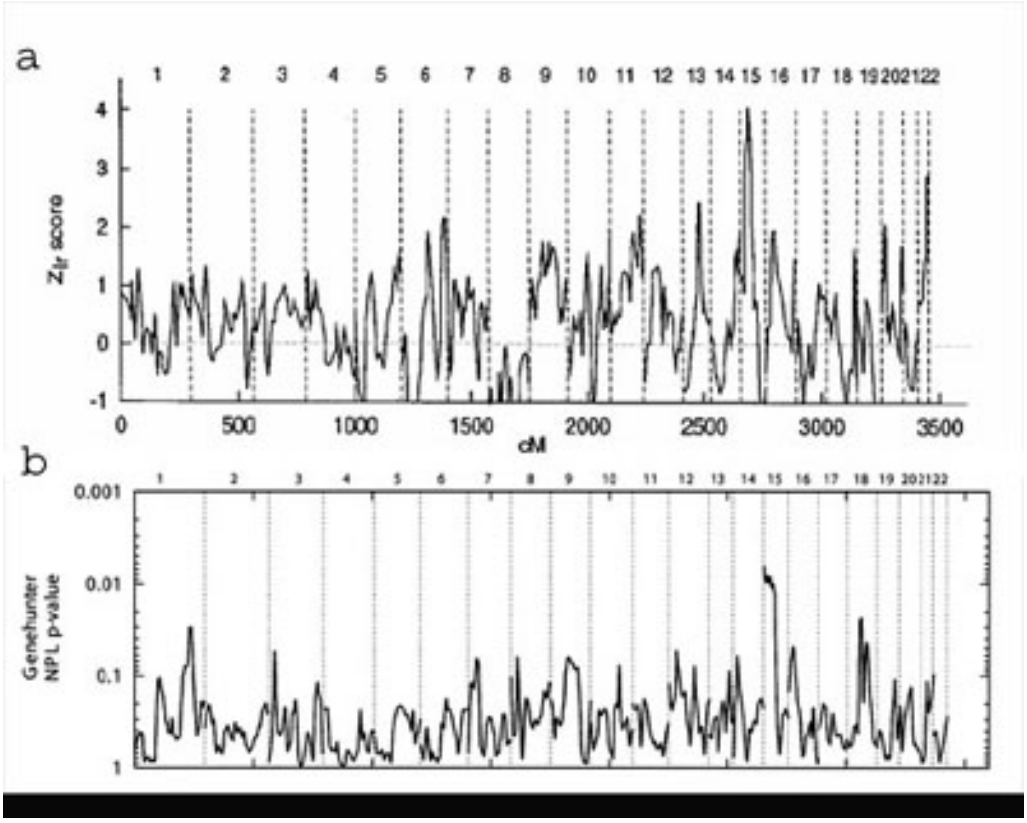


Figure 1. Genome-wide scan studies for German pedigrees with periodic catatonia produced linkage signal on chromosome 15 (taken from Stöber et al., 2001 and 2002).A. The first genome-wide scan study gave signals in chromosome 15 and additionally in chromosome 22. B. The second genome-wide scan study gave signal again in chromosome 15.

The study on susceptibility loci 15q and 22q in this thesis will be discussed in two parts. The first part, fine mapping of schizophrenia susceptibility locus in 15q14-15.1, will discuss the result on fine mapping work on chromosome 15q. In the current study, we have

saturated the interval between markers D15S1042 and D15S659 to refine the borders. Genotype and haplotype analysis were done in order to see whether the borders of mapped region are shared by all pedigrees described in the previous genome-wide scan studies (Stöber et al., 2000, 2002). Furthermore, direct sequence analyses were performed to the interesting non-coding regions (ultra conserved elements) and to one of the candidate genes located within the susceptibility region, namely *SPRED1* (Sprouty-related, EVH1 domain containing 1) gene.

The second part will be on the work of refining the map of susceptibility locus in chromosome 22q13.33 and the journey to reveal the function of *MLC1* (megalencephalic leukoencephalopathy with subcortical cyst 1), a gene that resides in the corresponding mapped region. This work is based on a report of a rare variant exclusively co-segregating with psychosis in a large pedigree (Meyer et al., 2001) included in the genome wide scan study of interest. At that time, the *MLC1* gene was publicly announced as the first gene ever found being involved in familial schizophrenia (Mirnics & Lewis, 2001; Wong, 2001).

1.3 Fine Mapping of Schizophrenia Susceptibility Locus in 15q14-15.1

Analysis of combined NIMH Schizophrenia data using a dominant model revealed convincing evidence for linkage to 15q (Freedman et al., 2001a). Reanalysis of the same data that calculated heterogeneity LOD (HLOD) recently revealed highly suggestive evidence of linkage to chromosome 15q14 again, with the addition of significant evidence of linkage to chromosome 10p that was not detected before (Holliday et al., 2005).

In a genome-wide linkage scan, Stöber and colleagues (2000) found evidence for a major susceptibility locus on chromosome 15q15 in most of the pedigrees analyzed

(maximum nonparametric LOD score of 3.57) for periodic catatonia. One of candidate genes in this region was *SLC12A6*. Further mutational analysis on *SLC12A6* in one multiplex pedigree (F11) revealed two rare variants in the promoter and 5´-UTR that co-segregate with the disorder. These variants were in linkage disequilibrium with each other and significantly associated with bipolar disorder in a case-control study (Meyer *et al.*, 2005). A methylation leading to reduced promoter activity was found later for one of the variants in the *SLC12A6* promoter (Moser *et al*., 2008). However, these rare variants were not found in other pedigrees from the study above.

In the second genome-wide scan, Stöber et al. (2002) found the overlapping region with the previous genome-wide scan result on chromosome 15q and were able to refine the disease gene locus to an 11-cM region between D15S1042 and D15S659. The refinement excludes important brain-expressed genes, such as *CHRNA7*, *SLC12A6* and *RYR3*. Further genotyping using polymorphic markers around *CHRNA7* in these pedigrees also showed a recombination event leading to exclusion (Meyer *et al*., 2002, 2003), in contrast to the finding in pedigrees from the NIMH Schizophrenia Genetics Initiative (Freedman *et al*., 2001). A similar result was also found earlier by Curtis and colleagues (1999). They used three polymorphic markers near *CHRNA7* and found that the region around the *CHRNA7* locus did not contain a major locus for susceptibility to schizophrenia in their pedigrees.

The refinement done by Stöber and colleagues (2002) was only based on two large pedigrees. On the other hand, no causal variants were found in several candidate genes in the region defined by Stöber et al. (2002) so far. *DLL4*, *SLC30A4*, *EIF2AK4* and *GCN2* were already analyzed and excluded as candidate genes (Mc Keane *et al*., 2005; Stöber et al; Kury *et al*., 2003; and unpublished data). Therefore, in the current study, we saturated the interval

between markers D15S1042 and D15S659 to further refine the borders. In order to see whether the borders are also shared by other pedigrees, genotype and haplotype analysis were done for 11 pedigrees described in the previous study which showed linkage to 15q.

1.4 Haplotype analysis on chromosome 1q42 and chromosome 6q loci

Additionally, in the first genome-wide scan (Stöber *et al.*, 2000), positive nonparametric-linkage and LOD scores of $P < .01$ were also detected at several regions throughout the genome. These may represent spurious linkage or weak true-minor loci. Interesting regions were on chromosome 1 and chromosome 6. In the first genome-wide scan for our German pedigrees, there was a weak signal in chromosome 6. Kohn and Lerer (2005) identified 5 loci of positive findings on chromosome 6q and suggested that each may harbor gene(s) that confer susceptibility to schizophrenia or bipolar disorder. Although previous study provided evidence that *KCNN3* was not the causative gene in our familial schizophrenia subtype of periodic catatonia, several studies gave a replicable linkage signal in 1q42 locus (Hamshere et al., 2005; Macgregor et al., 2004; Hwu et al., 2003). The Disrupted in Schizophrenia 1 (*DISC1*) gene, on chromosome 1q42, was originally discovered and linked to schizophrenia in a Scottish kindred carrying a balanced translocation that disrupts *DISC1* and *DISC2* (Millar et al., 2000, 2004; Blackwood et al., 2001). More recently, *DISC1* was linked to schizophrenia, broadly defined, in the general Finnish population (Ekelund et al., 2001). Therefore, haplotype analysis on chromosome 1q42 and chromosome 6q loci were additionally done with markers D1S419, D6S1715, D6S292 and D6S311.

1.5 Linkage Analysis

The LOD (logarithm of the odds) score is a statistical estimate of whether two loci are likely to lie near each other on a chromosome and are therefore likely to be inherited together. A LOD score of three or more is generally taken to indicate that the two loci are close. The Lod score is calculated as follows:

$$LOD = Z = \log 10 \frac{\text{probability of birth sequence with a given linkage value}}{\text{probability of birth sequence with no linkage}}$$
$$= \log 10 \frac{(1-\theta)^{NR} \times \theta^{R}}{0.5^{(NR+R)}}$$

1.5.1 Parametric linkage analysis

Standard LOD score analysis is called parametric because it requires a precise genetic model, detailing the mode of inheritance, gene frequencies and penetrance of each genotype. As long as a valid model is available, parametric linkage provides a powerful method for scanning the genome in 20-Mb segments to locate a disease gene. For nonmendelian diseases, specifying an adequate model is much less tractable.

One solution to the problem of having to specify the penetrance in parametric linkage analysis is to use a parametric method but analyze only the affected pedigree members. The penetrance is irrelevant for affected people, and unaffected members are scored as having an unknown disease phenotype. If the penetrance is low, unaffected people provide relatively little information. This strategy is useful for testing candidate susceptibility loci for oligogenic diseases. Since a parametric analysis is used, it is still necessary to specify a genetic model and so there is still the danger of getting meaningless results if the model is wrong.

1.5.2 Nonparametric linkage analysis

If the need to specify a complete genetic model is too daunting, one can use model-free or nonparametric methods of linkage analysis. These methods ignore unaffected people, and look for alleles or chromosomal segments that are shared by affected individuals. Shared segment methods can be used within nuclear families (sib pair analysis, see below), within known extended families, or in whole populations.

It is important to distinguish segments identical by descent (IBD) from those identical by state (IBS). IBS alleles look the same, and may have the same DNA sequence, but they are not derived from a *known* common ancestor. Alleles IBD are demonstrably copies of the same ancestral (usually parental) allele. For very rare alleles, two independent origins are unlikely, so IBS generally implies IBD, but this is not true for common alleles. Multiallele microsatellites are more efficient than two-allele markers for defining IBD, and multilocus multiallele haplotypes are better still, because any one haplotype is likely to be rare. Shared segment analysis can be conducted using either IBS or IBD data, provided the appropriate analysis is used. IBD is the more powerful, but requires parental samples. Multipoint analysis is preferable to single-point analysis because it more efficiently extracts the information about IBD sharing across the chromosomal region. Several approaches are used in nonparametric linkage analysis, namely affected sib pair (ASP) and affected pedigree member (APM) methods.

In the affected sib pairs (ASP) method, a picked chromosomal segment at random, pairs of sibs are expected to share 0, 1 or 2 parental haplotypes with frequency 1/4, 1/2 and 1/4, respectively. However, if both sibs are affected by a genetic disease, then they are likely

to share whichever segment of chromosome carries the disease locus. Because sib pair analysis is model-free, it can be performed without making any assumptions about the genetics of the disease. Thus, it has been used as one of the main tools for seeking genes conferring susceptibility to common nonmendelian diseases like diabetes or schizophrenia. One drawback is that candidate regions defined by sib pair analysis are usually uncomfortably large for positional cloning.

The affected pedigree member (APM) method of Weeks and Lange (1992) extends the logic of affected sib pair analysis to other relationships. In a complex pedigree with several affected people, for each pair of affected pedigree members the distribution of alleles identical by state is observed, and compared to the expectation on the null hypothesis of no linkage. APM allows multipoint data to be analysed in large pedigrees; however, because it uses IBS and not IBD data, it does not necessarily use all the linkage information that could in theory be extracted from a pedigree.

A more radical approach to nonparametric analysis of complex pedigrees is implemented in the genehunter program of Kruglyak et al. (1996). This is based on a generalization of the mapmaker/sibs program for analysis of multipoint ASP data mentioned above. The basic algorithm in these programs is able to handle any number of loci (the computing time increases linearly with the number of loci), but is limited to fairly small pedigrees. Pedigrees contain founders (people whose parents are not included in the pedigree) and nonfounders (people whose parents are included). If somebody has a sib in the pedigree, then they must be nonfounders, because the only way to tell the computer that they are sibs is to include the parents. If a pedigree contains f founders and n nonfounders, the

genehunter computing time increases exponentially with ($2n$ - f). Current versions fail to cope with pedigrees where $2n - f > 16$.

Provided a pedigree falls within the size limit, genehunter can include any number of loci in a multipoint analysis. It is in fact able to compute parametric lod scores, if a concrete genetic model is provided. For complex characters where no model can be provided, the result is expressed as a nonparametric lod (NPL) score. These are based on calculating the extent to which affected relatives share alleles identical by descent, and comparing the result across all affected pedigree members with the null hypothesis of simple mendelian segregation (markers will segregate according to mendelian ratios unless the segregation is distorted by linkage or association). This method appears to extract the linkage information from a pedigree more efficiently than the APM method. However, the threshold of significance for a NPL is not as obvious as with the parametric lod score. The significance is best expressed as a genome-wide p value.

Lander and Kruglyak (1995) defined "suggestive evidence" as a LOD score >2.2, which is equivalent to an NPL score >3.18 (Table 1). One should have an NPL score >4.08 and a P value <.000022 before claiming to have significant evidence of linkage.

Level	*P*	LOD	NPL
"Potentially interesting"[a]	.023	.87	2.00
Suggestive	.00074	2.2	3.18
Significant	.000022	3.6	4.08
Highly significant	.0000003	5.4	4.99

[a] As defined by Chen et al. (2004, p. 881).

Table 1. Comparison of NPL and LOD scores on the basis of Criteria from Lander and Kruglyak (1995), defined by Chen et al. (2004).

1.6 Sequence analysis of ultra conserved elements in locus 15q14-15.1

The human and mouse genomes share a number of long, perfectly conserved nucleotide sequences, termed ultraconserved elements. Whereas these regions can act as transcriptional enhancers when upstream of genes, those within genes are less well understood. There are 481 segments longer than 200 base pairs (bp) that are absolutely conserved (100% identity with no insertions or deletions) between orthologous regions of the human, rat, and mouse genomes. Nearly all of these segments are also conserved in the chicken and dog genomes, with an average of 95 and 99% identity, respectively. Many are also significantly conserved in fish.

The ultraconserved elements of the human genome are most often located either overlapping exons in genes involved in RNA processing or in introns or nearby genes involved in the regulation of transcription and development (Bejerano *et al.,* 2004). In particular, the function of ultraconserved elements that overlap alternatively spliced exons of genes encoding RNA-binding proteins is unknown.

Some of these ultraconserved elements were proved to be functional (Spitz *et al.*, 2003; Lettice *et al.*, 2002), and some were not (Nobrega *et al.*, 2004). Therefore, it is

interesting to analyze the ultraconserved elements that reside in our chromosome 15-susceptibility locus.

1.7 *SPRED1*, a candidate gene at chromosome 15q14

Spred1 is a member of the Sprouty /SPRED family of proteins that regulate growth factor-induced activation of the MAP kinase cascade (Nonami et al., 2004). The deduced 444-amino acid protein contains an N-terminal Enabled/VASP homology-1 (EVH1) domain, a central KIT-binding domain (KBD), and a C-terminal SPRY domain. Mammalian Spred1 is phosphorylated in response to several growth factors, and efficient phosphorylation required the KBD domain (Wakioka et al., 2001). Overexpression of Spred1 inhibited NGF-induced neurite differentiation in rat PC12 cells. Wakioka et al. (2001) presented evidence that Spred1 and Spred2 could regulate differentiation in rat neuronal cells and mouse myocytes by inhibiting activation of MAP kinase. Spred1 and Ras formed a complex which could inhibit activation of MAP kinase by suppressing phosphorylation and activation of Raf protein.

Mitogen-activated protein kinases (MAPKs) are important mediators of signal transduction from the cell surface to the nucleus and have been implicated in the integration of a variety of physiologic processes in most cells, including neurons. It is known that mitogen-activated protein kinase (MAPK) signaling pathways also respond to dopaminergic and serotonergic agents and mediate short- and long-term effects of intracellular signaling in neurons. Kyosseva and colleagues (1999 and 2004) performed a comparison of MAPK intermediates level in postmortem brain tissue obtained from schizophrenic and control subjects. They found that MAPK levels are elevated in the cerebellar vermis of schizophrenic

subjects and might be involved in the pathology of the disease. Importantly, Brown and colleagues (2005) showed that the antipsychotic agent, clozapine, selectively activates the MEK/ERK MAPK pathway in rat prefrontal cortex, and thus supported the hypothesis of MEK/ERK signal transduction cascade participation in clozapine's antipsychotic actions.

Involvement of SPRED1 in MAP kinase cascade made it as one of schizophrenia candidate genes in our locus that worth for further investigation.

1.8 Fine Mapping of Schizophrenia Susceptibility Locus in 22q13.33

In a genome-wide linkage scan, Stöber and colleagues (2000) found evidence for a major susceptibility locus on chromosome 15q15 in most of the pedigrees analyzed (maximum nonparametric lod score of 3.57), and a further potential locus meeting criteria for suggestive evidence for 22q13.33, pointing to genetic heterogeneity in periodic catatonia. This chromosomal locus, 22q13.33, was supported mainly by a single large pedigree. Genotyping of additional chromosome 22-specific polymorphic markers narrowed the region of interest to the D22S1160-telomer interval spanning about 4 centiMorgan.

Based on a report of a rare variant exclusively co-segregating with psychosis in a large pedigree (Meyer et al., 2001), the *MLC1* gene was publicly announced as the first gene ever found being involved in familial schizophrenia (Mirnics & Lewis, 2001; Wong, 2001). Shortly after, the finding of the same gene being causative for a severe neurodegenerative disorder, megalencephalic leukoencephalopathy with subcortical cysts (MLC) shed doubt on its putative role in pathogenesis of periodic catatonia type schizophrenia (Leegwater et al., 2001, 2002a, 2002b).

Meyer and colleagues (2001) described a rare polymorphism, Leu309Met, in the *MLC1* gene. The methionine-encoding variant, which was not present in the controls, was found co-segregating with the syndrome in the large pedigree that supports the 22q13.33 locus. While several other studies reported that the Leu309Met polymorphism did not exist in their samples (Kaganovich *et al*., 2004; Leegwater *et al*., 2002; Devaney *et al*., 2003, McQuillin *et al*., 2002), Rubie and colleagues (2003) found the rare methionine-encoding variant again in a single German patient with periodic catatonia. In their study, the rare variant was not present in 270 healthy blood donors and additional 140 index cases. The authors stated that some of the patient's relatives also suffered from psychoses, but were not carriers of the methionine-encoding variant. Thus, they formally excluded *MLC1* as a putative cause of periodic catatonia.

In a recent paper, Verma and associates (2005) stated that they have found three other rare *MLC1* polymorphisms, i.e. Val210Ile, Leu308Gln, and Arg328His, in six subjects from a patient sample with manic-depressive disorder from India. Of these, only Val210Ile was also found in a single subject from the healthy controls. Considered the close relationship between periodic catatonia and bipolar disorder (Taylor and Fink, 2003), the finding of Verma and colleagues further supports the involvement of *MLC1* in pathogenesis of psychosis. One of the polymorphisms in the Indian population, Leu308Gln, is intriguingly located in the polyleucine motif present in the putative S6 region of the protein, adjacent - only two base pairs upstream on DNA level - to the Leu309Met-encoding polymorphism described by our group (Meyer et al., 2001) and therefore strengthens the importance of this gene in psychiatric disease.

The finding of the 309Met-encoding allele in a new single German pedigree with

periodic catatonia by Rubie and colleagues (2003) is intriguing, considering the absence of this variant in all samples throughout the world tested for it so far, and even in Germany. Although they formally excluded *MLC1* as a putative cause of periodic catatonia in this pedigree, it is very interesting that the pedigree in question, namely F20, also originates from the same German region where the first pedigree described to carry the mutation, F21, lives. Thus, a founder effect for presence of this variant in the two pedigrees must be assumed.

1.9 *MLC1* as candidate gene

MLC1 (OMIM #605908) is the official symbol provided by the HUGO Nomenclature Committee for a gene originally described by Nomura and colleagues as *KIAA0027* - another synonym is *WKL1* - in their efforts to isolate large brain-specific transcripts (Nomura et al., 1994). *MLC1* mRNA is highly expressed in the brain, including cortex, hippocampus, caudate nucleus, amygdala, thalamus and cerebellum (Meyer *et al*., 2001), but also in leukocytes. Schmitt and associates (2003), and Boor and coworkers (2005) reported that *MLC1* is expressed in glial cells such as astrocytes, Bergmann glia and ependymal cells. Teijido and colleagues (2004) revealed that the gene is also expressed in neurons by in situ hybridization using double-labelled *MLC1* mRNA and the protein NeuN. Expression of mRNA was also detected in neuronal cell bodies of several brain regions, including pyramidal neurons in the neocortex, granule cells in the hippocampus, and in several midbrain and brainstem nuclei.

The function of the MLC1 protein is still unknown; however, weak similarity to other proteins suggests that it may be an integral membrane transporter (Boor *et al*., 2005). MLC1 protein is predicted to have 8 transmembrane domains by most of transmembrane domain

prediction computer programs. Recently, Boor *et al*. (2005) concluded that there are 8 transmembrane domains with both NH4+ and COOH- termini of MLC1 probably located in the cytoplasm. Interestingly, MLC1 also shows a similar localization to Aquaporin-4, a water channel protein present in the brain, and involved in distal astrogial processes (Badaut *et al*., 2000 and 2003, Boor *et al.*, 2005). While Boor and colleagues (2007) concluded that MLC1 is associated with the dystrophin-glycoprotein complex (DGC) at astrocytic endfeet, Duarri and associates (2008) were in disagreement and showed that MLC1 is located in astrocyte-astrocyte junctions, and its localization in several knock-out mouse models of DGC complex proteins was not altered.

Previously, it has been suggested that MLC1 is possibly a non-selective cation channel, which exhibits weakly structural similarity to the voltage-gated potassium channel Kv1.1 (Meyer *et al*., 2001, Leegwater *et al*., 2001). In the controversy of *MLC1* involvement in MLC or periodic catatonia, it is important to note that a missense mutation, Val408Arg, residing near the cytoplasmatic end of transmembrane domain S6 of Kv1.1, which is encoded by the gene *KCNA1*, is involved in another movement disorder, namely episodic ataxia type 1 (Herson et al., 2003).

1.10 Protein binding partner identification by yeast two-hybrid system

Attempts to detect ion channel activity of MLC1 have not been successful so far (Kaganovich et al., 2003, Teijido et al., 2004). However, there is a possibility that this protein could function via interaction with so far unknown ligands. Therefore, further attempts to identify the proteins that bind to MLC1 protein are very important. One technique to

elicidate the function of a protein is via determining its binding partner, in this case another protein.

Yeast two-hybrid screening or 2-hybrid screening is a molecular biology technique used to discover protein-protein interactions and protein-DNA interactions by testing for physical interactions (such as binding) between two proteins or a single protein and a DNA molecule, respectively. The premise behind the test is the activation of downstream reporter gene(s) by the binding of a transcription factor onto an upstream activating sequence (UAS). For the purposes of two-hybrid screening, the transcription factor is split into two separate fragments, called the binding domain (DB) and activating domain (AD). The DB is the domain responsible for binding to the UAS and the AD is the domain responsible for activation of transcription.

As depicted in Figure 2, in general, in any two-hybrid experiment a protein of interest is fused to a DNA-binding domain and transfected in a yeast host cell bearing a reporter gene controlling this DNA-binding domain. When this fusion protein cannot activate transcription on its own, it can be used as “bait” or as a “target” to screen a library of cDNA clones that are fused to an activation domain. The cDNA clones within the library that encode proteins capable of forming protein-protein interactions with the bait are identified by their ability to cause activation of the reporter gene. Thus, the yeast two-hybrid system is devised to identify genes encoding proteins that are physically associated with a given protein *in vivo*.

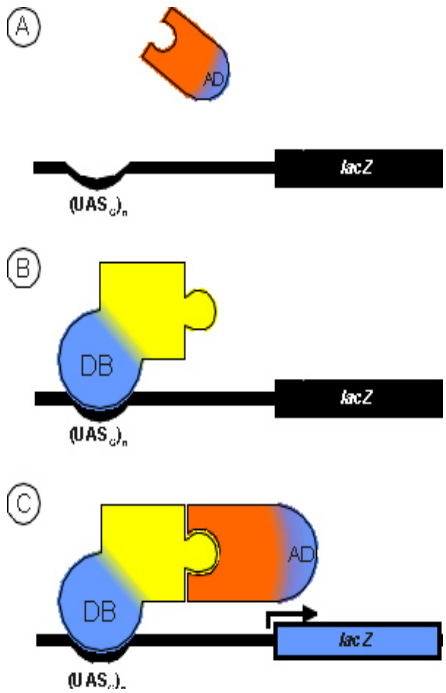


Figure 2. Principle of the Two-hybrid system (taken from Van Criekinge and Beyaert, 1999). (A), (B) Two chimeras, one containing the DNA-binding domain (DB) and one that contains an activation domain (AD), are co-transfected into an appropriate host strain. (C) If the fusion partners interact, the DB and AD are brought into proximity and can activate transcription of reporter genes (here LacZ).

To date, only one study reported that MLC1 weakly binds DISC1 in yeast two hybrid (Millar et al., 2003). In this study, DISC1 protein is used as bait. The disrupted in schizophrenia 1 (*DISC1*) gene has been identified as a candidate for schizophrenia and affective disorders, since a balanced 1:11 translocation disrupts the gene in a large pedigree. Carriers of the translocation suffer either of schizophrenia or affective disorder (Blackwood et al., 2001). Convergent data implicate DISC1 in genetic risk for schizophrenia and DISC1 seems to have a key role in building the brain and memories by interacting with other proteins (Harrison and Weinberger, 2005; Porteous and Millar, 2006). Therefore, the weak result of MLC1-DISC1 interaction from yeast two-hybrid technique should be further confirmed.

1.11 Knock-out mouse for *MCL1*

A knockout (KO) mouse is a genetically modified mouse which has one or several genes made inoperable by the insertion of a mutated gene or a disrupted form of the gene. The generation of knockout mice is important for the analysis of the function of many unknown genes.

Genetic susceptibility to common psychiatric disease arises from the complex interactions between a multitude of genes and an unknown number of relevant environments. However, a common method for investigating gene function involves the creation of a mouse knockout of a candidate gene. Although this approach seems inappropriate to model such complexity, genetic effects on behavior attributable to null mutants in the mouse are in fact subject to the same set of complications, the same gene by environment and epistatic interactions that characterize genetic effects in psychiatric illness (Flint, 2006). Recently, Carpenter and associates (2007) compiled a table for the animal models of schizophrenia, where 59% from 61 animal models were knockouts.

Considering the findings in MLC and periodic catatonia involving *MLC1*, it will be important to not only characterize the knockout mouse from a morphometric, biochemical and neurodevelopmental point of view, but also to see the effects to behavior and sensorimotor gating, which is disrupted in schizophrenia. Thus, the knockout will allow us to understand the bases of the clinical symptoms of the disease for MLC as well as periodic catatonia.

1.8 Research Goal

The goals of this study are 1) To saturate the interval between markers D15S1042 and D15S659 to refine the susceptibility region and performed genotype and haplotype analysis for 11 pedigrees described in the previous study from Stöber et al. (2000) in order to see whether other pedigrees share, or narrow down the borders. Furthermore, 2) to sequence the 15q15 ultra-conserved elements and *SPRED1* that are located within the susceptibility region and additionally to performe haplotype analysis for loci at chromosome 6q by genotyping satellite markers D6S1715, D6S292, and D6S311. For chromosome 22q13 locus, 4) to refine the mapping region in the pedigree 20 and to prove that *MLC1* could not be excluded as periodic catatonia candidate gene. Finally, a preliminary step from our group towards elucidating the function of *MLC1* in this study is 5) the construction of mouse knockout vector for *MLC1* and the yeast-two hybrid approach to identify the protein binding partner.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Sample collection

Members of large pedigrees were ascertained as previously described in detail by Stöber et al. (2000 and 2001), including the DNA isolation from the blood samples. All pedigrees were depicted in Figure 3. Eleven multiplex pedigrees from the first genome-wide scan study consisting of 113 individuals (50 affected with DNA specimen available) were included for present study.

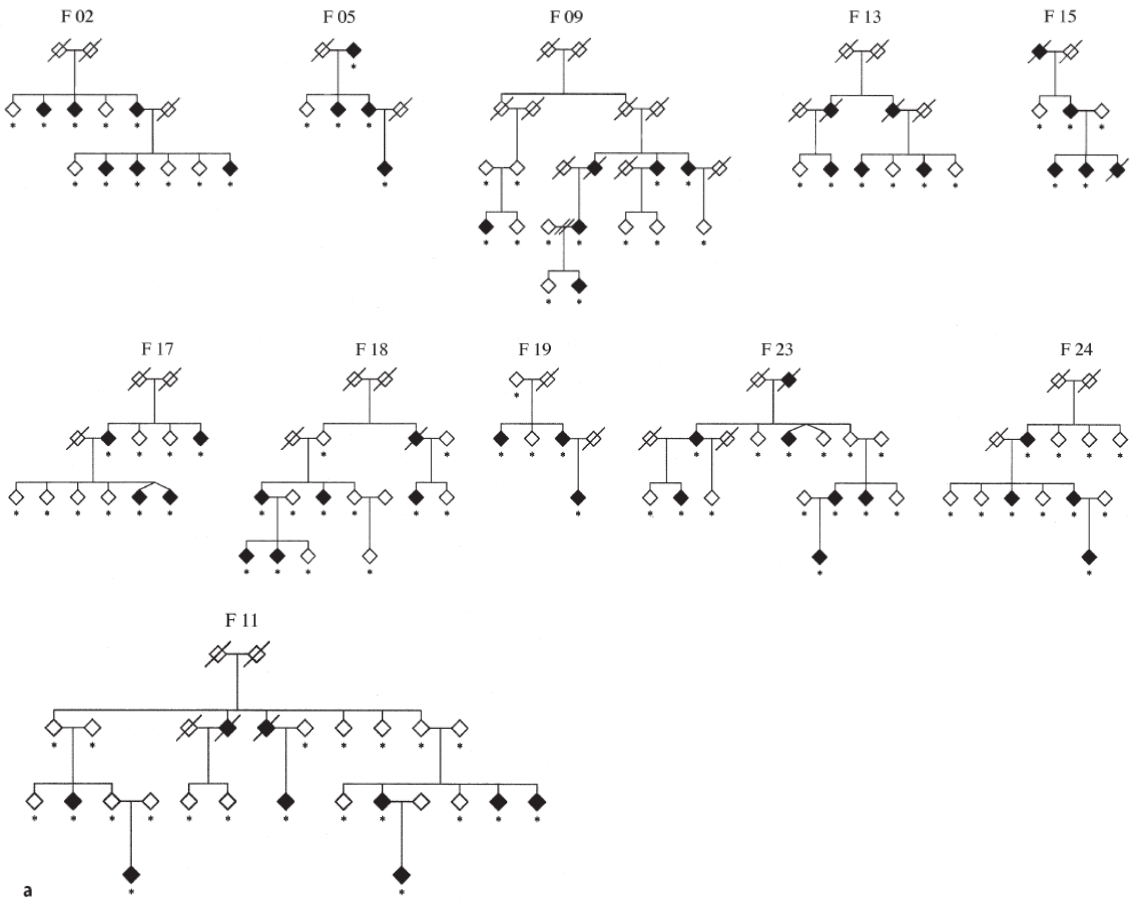


Figure 3. German multiplex pedigrees segregating periodic catatonia included in this study for linkage analysis in chromosome 15 (taken from Stöber et al., 2001). A slash through the symbol indicates that the individual is deceased. Open symbols represent unaffected individuals, solid symbols represent individuals with final diagnosis of periodic catatonia. A star indicates those individuals with DNA specimen available.

2.1.2 Oligonucleotides

Oligonucleotides used in chromosome 15 fine-mapping, mutation analysis of SPRED1 and ultraconserved region are listed in Table 2 below.

Primer	Location	Sequence (5´ to 3´)	Ánnealing	Amplicon size (bp)
Chromosome 22				
trier7	Chr22: 48547157-48547413	Forward :AACACGGCCAAATCTCAAAC	56 °C	256
		Reverse :GAGGGTAGAATCAACTCCATGC		
trier8	Chr22: 48712248-48712545	Forward :GATCACGGCAGTACAGTCCA	60 °C	297
		Reverse :GCTCCATATTGTCCATTTGGTT		
trier9	Chr22: 48735037-48735308	Forward :GCTTACTGCTTCCCATTTGC	58 °C	271
		Reverse :GCAGAACACCCCAGCTAGAC		
trier10	Chr22: 49123743-49124028	Forward :GCGGCAAAGTGAGATTCTGT	58 °C	285
		Reverse :CCCCTTCTTGTCCAGTTTCA		
trier11	Chr22: 49468549-49468847	Forward :TACCCGATTGGCTGTTTGTT	57 °C	298
		Reverse :GCACTGTGGAAAACTTTGAGG		
Chromosome 15				
UC 382	chr15:33634968-33635167	Forward :GAAGCCATGTACAATCCGTCT	58 °C	299
		Reverse :GGAGGAATGGAAAGGAGAAA		
UC 383	chr15:34535983-34536251	Forward :TCATGCAAATTACACCAGAGC	58 °C	340
		Reverse :TGGTCAAATCTGTGTTTCACATC		
UC 384	chr15:34681449-34681714	Forward :TCAAATGAGGGCTTTAGCAG	58 °C	343
		Reverse :TGCAGGAGGAATGCAATAAA		
UC 385	chr15:34901726-34901934	Forward :AACAGAAGTGGCAAAACCATC	59 °C	277
		Reverse :AAAACATTTGCTCAGCTTGGA		
UC 386	chr15:35238065-35238267	Forward :TGATTCCGTTCTTTGCTGTG	58 °C	257
		Reverse :TCAGATAATGCTGCAGGACA		
UC 387	chr15:39748110-39748347	Forward :AAAAATGAAGCCCCTGTCAA	59 °C	305
		Reverse :GCCATTTTGGAATTGGATGT		
SPRED1				
SPRED1	predicted promoter and exon1	Forward :CTACTTCGCCTCCTTCTCTC	62.5 °C	357
		Reverse :GCGGATTCCTCCAGACAC		
	exon 2	Forward :GTACCGTTCTGGGTGAGG	56 °C	340
		Reverse :AAATGGCTAAAGAAATCAGC		
	exon 3	Forward :AACAAGACTGATGGCTTGG	56 °C	375
		Reverse :ACACAGAAACAGCTCCAGAA		
	exon 4	Forward :GTTGTATCACCTCAGTTT	56 °C	390
		Reverse :TCAAAGCCTGGTCACATATC		
	exon 5	Forward :CTCGTTAGTAAGCAGCTGGA	56 °C	407
		Reverse :TTCTTCTGGAGAAATCTGACA		
	exon 6	Forward :TTGCTATTCATAGCGATGGT	56 °C	393
		Reverse :GTGTCTGGTAAAGGGCAGAT		
	exon 7	Forward :TGAGGTTTTGGAACATACACT	55 °C	386
		Reverse :CAGCTCTGGCAATCTTTTAG		
	8 cds part1	Forward :CAACAAATATCTGGACACTGG	55.5 °C	473
		Reverse :GAGCATCCTGACATTTTCC		
	8 cds part2	Forward :ACTGCCAGGAAAGGTTTAAT	55.5 °C	394
		Reverse :TTCCACAAATCCAAGTTAGC		
	8 utr part1	Forward :CTGTGGTGGGAAACATAAAG	55.5 °C	481
		Reverse :AGTTGCACTTGTTTTACTTC		
	8 utr part2	Forward :AAAGTGCACTAGGGGACAG	57 °C	633
		Reverse :CAGTCAAACTTTCTGCCTTG		
	8 utr part3	Forward :TCAGAGTTTCCTCCTTCAAA	55 °C	882
		Reverse :AAATACCCTCTTCTTCATAACA		
	8 utr part4	Forward :TTTCACATTGAAAAGAAGAAAA	55 °C	656
		Reverse :TCCAAGGTAAAATAAAAACCAT		

Table 2. PCR primer sequences.

2.1.3 Molecular biology kits

Kit	Manufacturer
Big Dye® Terminator v.1.1. Cycle Seqencing	PE Applied Biosystems, Weiterstadt
dNTPs (ATP, TTP, CTP, GTP)	Fermentas, St. Leon Roth
Min Elute™ PCR Purification Kit	QIAGEN, Hilden
Min Elute™ Gel Extraction Kit	QIAGEN, Hilden
QIAamp® DNA Micro Kit	QIAGEN, Hilden
Wizard® DNA Clean-up System	Promega, Mannheim
Gene Ruler™ 100bp DNA Ladder Plus	Fermentas, St. Leon Roth

2.1.4 Reagents and Chemicals

Chemicals	Manufacturer
Agarose Roti®garose	Fa. Roth , Karlsruhe
Ammonium acetate	Sigma-Aldrich, Deisenhofen
Bovine Albumin Serum (BSA)	New England Biolabs
Bromphenol blue-Na-Salt (BPB)	Serva, Heidelberg
Dinatrium methylen diaminteraacetate (EDTA)	Merck, Darmstadt
Dimethylsulfoxide (DMSO) (C2H6OS)	Biochrom, Cambrige/UK
Ethidium bromide	Fa. Roth , Karlsruhe
Ethanol	Fa. Roth , Karlsruhe
Ficoll 400	Promega, Mannheim
Glycerin	Amresco®, Ohio/USA
Isopropanol (C3H8O)	Fa. Roth , Karlsruhe
Natrium acetate (C2H3NaO2)	Fa. Roth , Karlsruhe
Natrium chloride (NaCl)	Fa. Roth , Karlsruhe
Natrium hydroxide (NaOH)	Fa. Roth , Karlsruhe
Magnesium chloride	Fa. Roth , Karlsruhe
Mineral oil	PE Applied Biosystems, Weiterstadt
PIPES	Fa. Roth , Karlsruhe
TBE Puffer (10X) Rotiphorese®	Fa. Roth , Karlsruhe
Tris HCl (C4H11NO3)	Fa. Roth , Karlsruhe
Tween® 20	Fa. Roth , Karlsruhe
Xylen cyanol	Serva, Heidelberg

2.1.5 Instruments

Instrument	Manufacturer
ABI PrismTM-310 Genetic Analyzer	PE Applied Biosystems, Weiterstadt
AvantiTM Centrifuge J-25 I	Beckmann, München
Biofuge 13	Heraeus Sepatech GmbH, Osterode
Electrophoresis Power Supply-EPS200	Pharmacia Biotech, Freiburg
ELISA-Reader MR 7000	Dynatech, Denkendorf
Gel tank LKB GNA 100	Pharmacia Biotech, Freiburg
Gene Quant 2 RNA/DNA Calculator	Pharmacia Biotech, Freiburg
Gene Amp® PCR-Sytem 9700	PE Applied Biosystems, Weiterstadt
Horizontal gel chamber Multiphor 2	Pharmacia Biotech, Freiburg
Microwave	AEG, Nürnberg
Multitemp 3 Waterbath	Pharmacia Biotech, Freiburg
PH-Meter 766 Calimatic	Knick, Berlin
Shake Incubator	Onkyo/Gallenkamp, UK
Thermomixer 5436	Eppendorf, Hamburg
Table centrifuge Z 233 M	Hermle, Wehingen
UV-lamp N -90M	INTAS UV – Systeme, Wiesloch
Vacuum Centrifuge (Speed-Vac-Concentrator)	Savant, Farmingdale, USA
Balance L420P	Sartorius Laboratory, Göttingen

2.1.6 Buffer

Components	PCR Buffer			
	A	B	C	D
Potassiumchloride (KCL)	500mM	500mM	500mM	500mM
Tris-HCl (pH 8,3)	100mM	100mM	100mM	100mM
Tween20	0,25%	0,25%	0,25%	0,25%
Bovine serumAlbumin (BSA)	0,25mg/ml	0,25mg/ml	0,25mg/ml	0,25mg/ml
Magnesiumchloride (MgCl2)	7,5mM	10,0mM	15,0mM	20,0mM

2.2 Methods

2.2.1 Mutation Analysis

We used *SPRED1* annotation provided from Ensembl (http://www.ensembl.org) and UCSC (www.genome.ucsc.edu, May 2004 freeze). Sequence data of satellite markers and ultra-conserved elements were also obtained from UCSC. Primer pairs encompassing the proximal promoter region, 5´-UTR, coding regions and intron/exon boundaries were designed using software Primer3 (Rozen and Skaletsky 2000, http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi, available upon request) and used to amplify genomic DNA from two affected members of pedigree 11 (patients 744 and 834), and one affected member of pedigree 9 (patient 568). We used PROSCAN 1.7 program online (http://thr.cit.nih.gov/molbio/proscan/) for promoter prediction.

Genomic DNA was amplified by polymerase chain reaction (PCR) in a final volume of 50 µl containing 100 ng genomic DNA, 10 pmol of each primer (MWG Biotech), 0.2 mM of each dNTP (MBI Fermentas), 7.5 to 15 mM $MgCl_2$, and 1 unit of Taq Polymerase (Eurogentec, Seraing, Belgium). Thermal cycling was performed using GeneAmp PCR System 9700 (Applied Biosystems). Amplicons were subsequently purified from agarose gel using the gel purification kit (PeqLab), according to the manufacturer's protocol. Direct sequencing was performed using an ABI Prism™ 310 Genetic Analyzer (Applied Biosystems) using the BigDye™ terminator Cycle Sequencing Kit following the manufacturer's protocol. Sequencing reactions were performed for both DNA strands. Sequences were analyzed using the ChromasLite program (Technelysium Pty Ltd). Resulting sequences results were aligned against reference sequences from the database (UCSC, May

2004 freeze) using Clustalw program available from European Bioinformatics Institute (www.ebi.ac.uk).

2.2.2 Genotyping chromosome 15q

Microsatellite markers were selected from the human genetic map (UCSC, May 2004 freeze). Twenty-five microsatellite markers were chosen for chromosome 15. The markers used in this study were D15S1035, D15S128, D15S165, D15S1007, D15S118, D15S1042, D15S1012, D15S126, D15S1033, D15S153, D15S114, D15S127, D15S130, D15S966, D15S194, D15S994, D15S968, D15S641, and D15S1044.

Additional six satellite markers were picked from the genomic annotation (UCSC, May 2004 freeze) and named Trier1 (Chr15:36136018-36136185), Trier2 (Chr15:36452796-36453006), Trier3 (Chr15:36622152-36622400), Trier4 (Chr15:36874542-36874916), Trier5 (Chr15:37160522-37160714) and Trier6 (Chr15:37413202-37413429).

Genomic DNA amplifications were done using labeled marker and PCR conditions previously described. Microsatellite markers were labeled with HEX, FAM or TAMRA dye (MWG Biotech). Depending on the intensity of the amplicon band, 2 or 3 µl of PCR product was mixed with 0.5 µl of 500 ROX™ standard (Applied Biosystems) and 12 µl Hi-Di formamide (Applied Biosystems). The mixes then were heated to 94°C for 2 minutes, and subsequently separated on ABI Prism™ 310 Genetic Analyzer automatic sequencers. Allele calling was done using the GenScan program (Applied Biosystems), and was checked by operational procedures by an operator blinded to phenotype

2.2.3 Linkage Analysis

A nonparametric multipoint linkage analysis for 11 pedigrees was calculated using the GENEHUNTER program.

Nonparametric, model-free multipoint linkage analyses were performed for chromosome 15-linked pedigrees (7 pedigrees) using SIMWALK program (Sobel and Lange 1996; Sobel et al 2002; Sobel et al 2001) with Madeline program version 0.935 (Trager, 2004, http://eyegene.ophthy.med.umich.edu/madeline-0.935/) as the interface. Nonparametric multipoint linkage was calculated for chromosome 15-linked pedigrees except F11 (6 pedigrees) using GENEHUNTER (Lander and Kruglyak 1995) through the EasyLinkage program (Hoffmann and Lindner 2005). Statistical significance was estimated based on shared alleles identical-by-descent (IBD) of all affected persons.

2.2.4 Mouse MLC1 knockout vector

The overview of the cloning strategy is depicted in Figure 4. A cosmid construct containing the mouse MLC1 genomic region (**MPMGc121H11670Q2, Steinke et al., 2002**) was digested with restriction enzyme PstI or SacI to generate the DNA fragments to be used further for constructing the knocknout vector.

A.

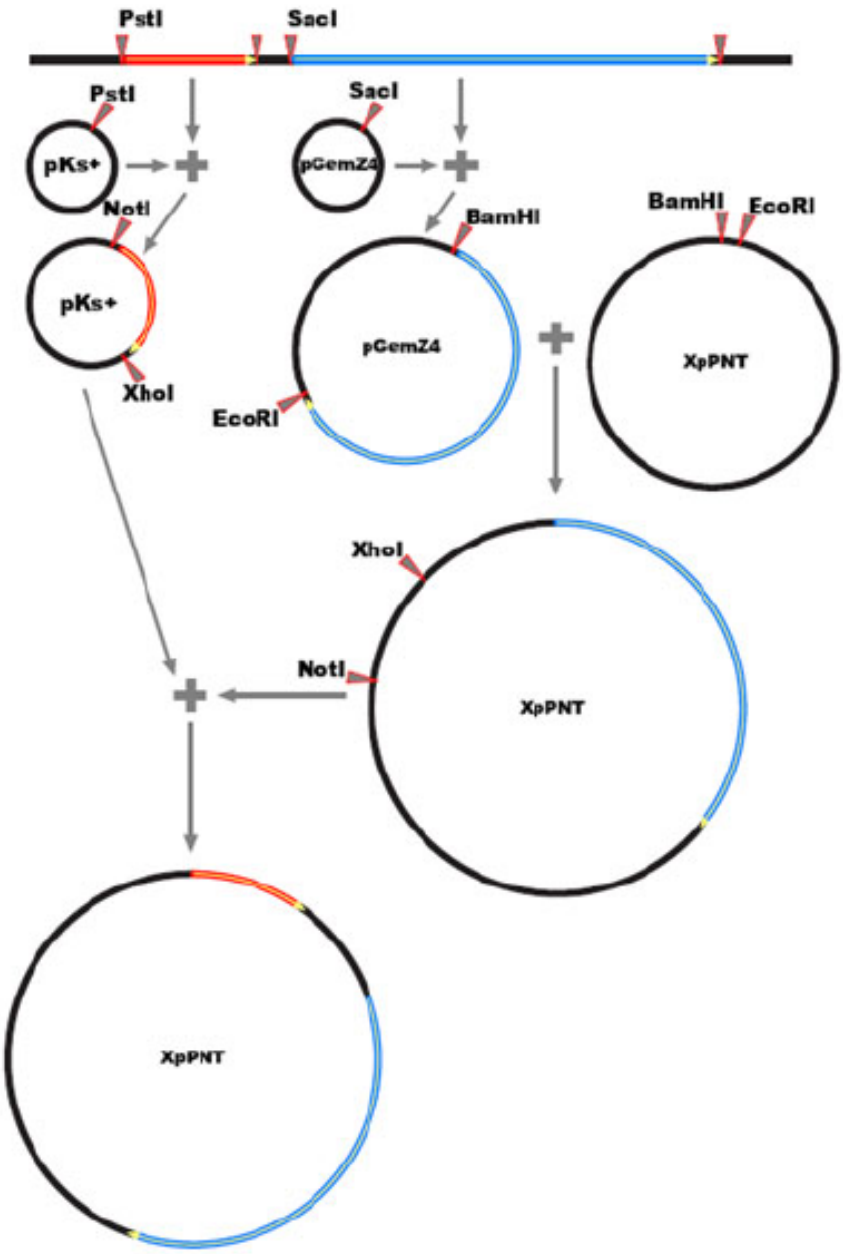


Figure 4. A) Mouse MLC1 knockout vector cloning strategy. Red bar represents the PstI fragment or left arm and blue bar represents the SacI fragment or right arm insert.

B.

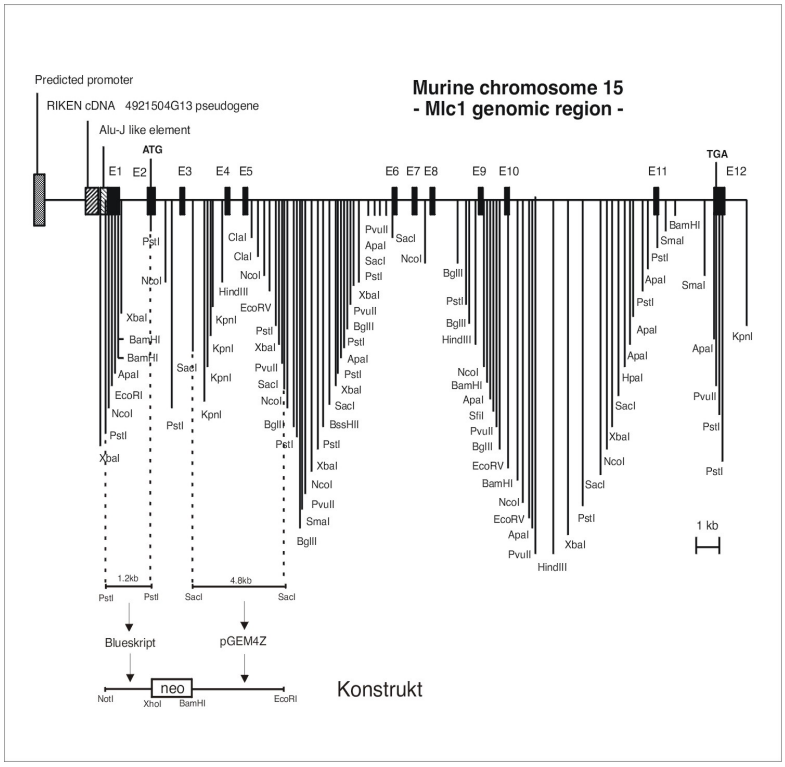


Figure 4. B) Generation of inserts for the left and right arm of the knockout vector (Meyer, unpublished).

The PstI-generated fragment(s) with the size of about 1.2 kb was ligated into the multiple cloning sites (MCS) of plasmid pKs+ already cut with PstI using T4 DNA ligase and used for the transformation of chemical competent cells *E.Coli* XL-1 blue. Screening of positive transformants was performed on LB agar with 50ug/ml ampicillin. A miniprep was then done to obtain DNA material from the positive transformants, and another restriction digest with PstI was performed to confirm the presence of inserts. DNA from clones with positive result from restriction digest were then submitted for DNA sequencing. Based on DNA sequencing results, a good clone with the correct orientation of the insert was selected for further cloning step into the knockout vector. The plasmid pKs+ provided restriction sites

of NotI and XhoI that then be used for cloning the PstI fragment into upstream of the neomycin cassette, or called the left arm, of the XpPNT knockout vector. This left arm contained exon 1 and part of exon 2 of mouse *MLC1* that will make the the frameshift mutation to *MLC1*, hence scrumbled protein.

The SacI fragment(s) with the size of about 4.8 kb containing *MLC1* exon 4 and exon 5 was cloned into plasmid pGEMZ4 with the same protocols aforesaid. The plasmid provided BamHI and EcoRI sites that then be used for cloning the SacI fragment into downstream of the neomycin cassette, or called the right arm. At the end, the left arm and right arm of the knockout vector will generate *MLC1* with exon 3 loss and frameshift mutation leading to the knockout of the gene.

2.2.5 Yeast Two Hybrid Bait for MLC1

For the construction of a hybrid protein bait, the gene encoding the protein of interest is ligated into the MCS of expression vector in the correct orientation and in the correct reading frame such that a fusion protein is generated. The bait vector used in this experiment was vector pGBT9, a shuttle vector that replicates autonomously in both *E. coli* and *S. cerevisiae*. It carries the *bla* gene (for ampicillin resistance in *E. coli*) and the TRP1 nutritional marker that allow yeast auxotrophs carrying pGBT9 to grow on limiting synthetic medium lacking Trp.

The hybrid construct of MLC1 in pGBT9 generates a hybrid protein that contains the sequences for the GAL4 DNA-binding domain (DNA-BD; a.a. 1–147). The fusion protein is expressed in yeast host cells from the constitutive ADH1 promoter; transcription is terminated at the ADH1 transcription termination signal. The hybrid protein is targeted to the

yeast nucleus by nuclear localization sequences that are an intrinsic part of the GAL4 DNA-BD.

A forward primer with EcoRI site tail and a reverse primer with SalI site tail were designed to be amplify *MLC1* coding sequence from human cDNA clone ha00522 (Kazusa, clone containing *MLC1* cDNA). PCR was performed in a 50uL total volume containing 20 ng plasmid DNA, 10 pmol of each primer (MWG Biotech), 0.2 mM of each dNTP (MBI Fermentas), 15 mM $MgCl_2$, and 1 unit of Taq Polymerase (Eurogentec, Seraing, Belgium). Thermal cycling was performed using GeneAmp PCR System 9700 (Applied Biosystems) with 5 min 95oC of first denaturation, followed by 30 cycles annealing (30 sec at 95oC, 45 sec at 61oC and 30 sec at 72oC), ended up with a final extension step at 72oC for 7 min. Forward primer was 5´-GAA TTC ACC CAG GAG CCA TTC AGA-3´ and reverse primer was 5´-GTC GAC CTG GGC CAT TTG CAC-3´.

Amplicons were subsequently purified from agarose gel using the gel purification kit (PeqLab), according to the manufacturer's protocol

The amplicon will be *MLC1* full length coding sequence lacking the start and stop codon, but having EcoRI site at the 5´-end and SalI site at the 3´-end, and will be the insert for later cloning into pGBT9 yeast two-hybrid bait vector.

The amplicon was then ligated into pGEM-T vector and the ligation products were used for competent *E.coli* XL-1 blue transformation. Miniprep DNA were done following screening of positive transformants on LB agar containing 50 ug/mL ampicillin. Positives clones DNA after insert confirmation with restriction digest were then submitted for DNA sequencing. A clone with the correct DNA sequence was then selected for DNA insert prepara-

tion through preparative DNA miniprep and preparative double restriction digest with EcoRI and SalI.

This insert DNA was then ligated into already EcoRI/SalI cut pGBT9 vector with T4 DNA ligase and used for competent *E.coli* XL-1 blue. Miniprep DNA from positive clones were then submitted for DNA sequencing. Finally, the *MLC1* bait construct with the correct sequences was sent to RZPD yeast two-hybrid service for transformation and automated screening (Albers et al., 2005).

Chapter 3

Results

This study is basically divided into 2 sub-studies, hence the following presentation of results and discussions. The first investigation will be on periodic catatonia locus 15q and the second one on locus 22q13.

For the investigation at locus 15q, the results will be of 1) mapping the chromosome region between markers D15S1042 and D15S659 to refine the susceptibility region and 2) performing genotype and haplotype analysis for pedigrees described in the previous study from Stöber et al. (2000). Furthermore, 3) the results from sequencing analysis of 15q15 ultra-conserved elements and *SPRED1* that are located within the susceptibility region, and 4) additional haplotype analysis for loci at chromosome 6q will also be presented below.

3.1 Haplotype analyses of 15q14-15

Haplotype analyses of 11 pedigrees revealed that seven of these pedigrees clearly showed linkage to chromosome 15q, whereas the remaining four pedigrees showed no linkage. All chromosome 15-linked pedigrees share a core candidate region, including one pedigree taken from the literature (F30; Stöber et al., 2002). A summary of these results is presented in Figure 11, while details from all pedigrees are depicted in Figures 5 to 10. It is to be noted that haplotype analysis for pedigree F9 was taken from previous unpublished fine-mapping results from Meyer et al. (2003).

We refined the 11 cM candidate region on 15q15 by defining the linked haplotypes of pedigree F11 and six other pedigree (F5, F9, F13, F17, F19, and F24) that show linkage to this region. This procedure delineated four sub-regions (A, B, C, D) of the SCZD10 locus (Figure 9). The most interesting sub-regions are sub-region B (shared by all 7 pedigree), and to a lesser extent region C (shared by 6 pedigree) at locus 15q14-15q15.1.

Three pedigrees (F5, F9 and F24) confirmed marker D15S1042 as the centromeric border, and pedigree F19 confirmed marker D15S968 as the telomeric border of sub-region B (4.3 megabases, Mb). The telomeric border of the small sub-region C, encompassing 1.2 Mb distally of D15S968, is defined by marker D15S641 in pedigree F9.

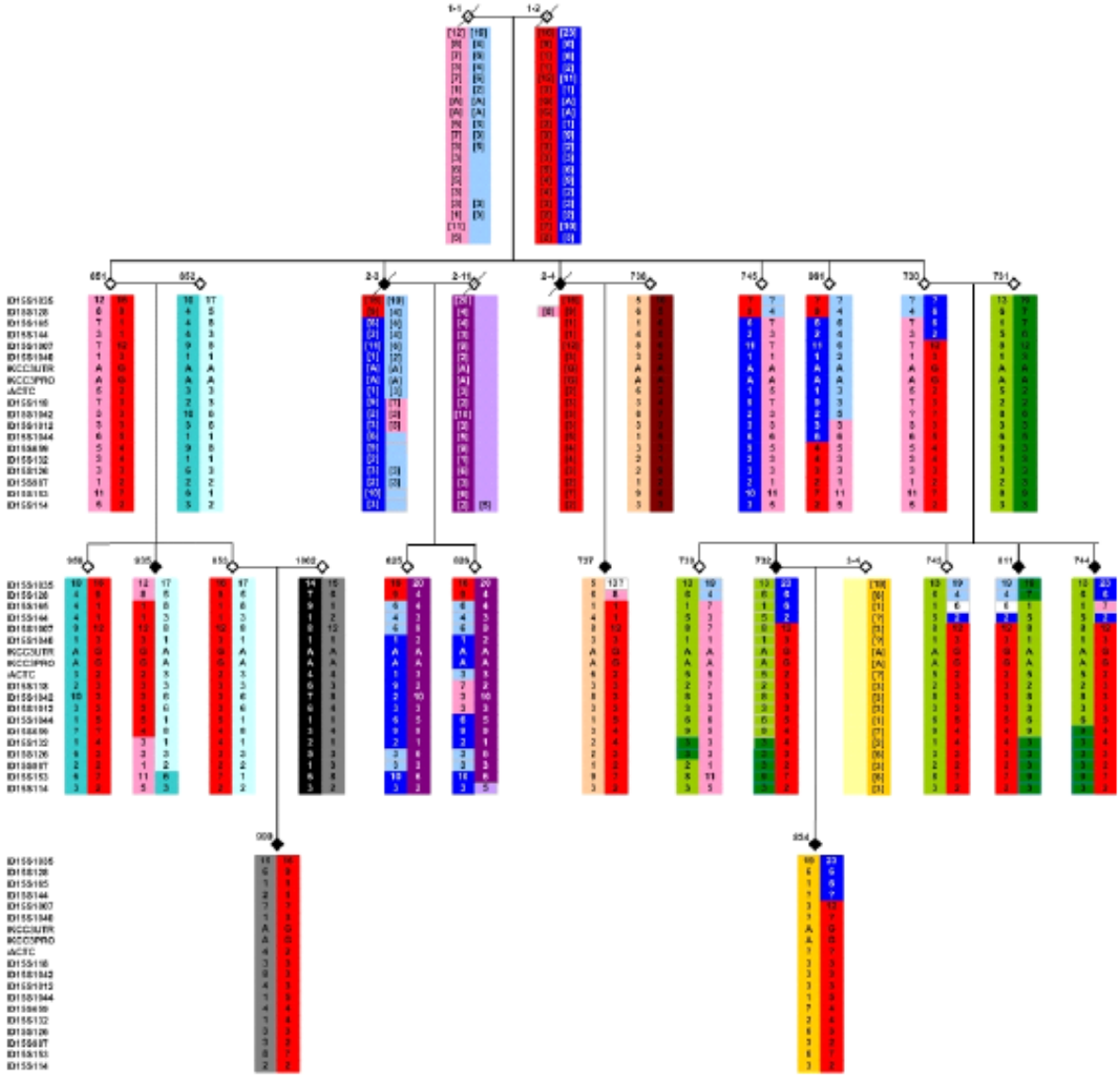


Figure 5. Haplotype analysis of pedigree F11. Black diamond represent the affected person, open diamon represent unaffected person. The red bar represent the linked haplotype block. The diagonal line across the diamond represent the ceased person.

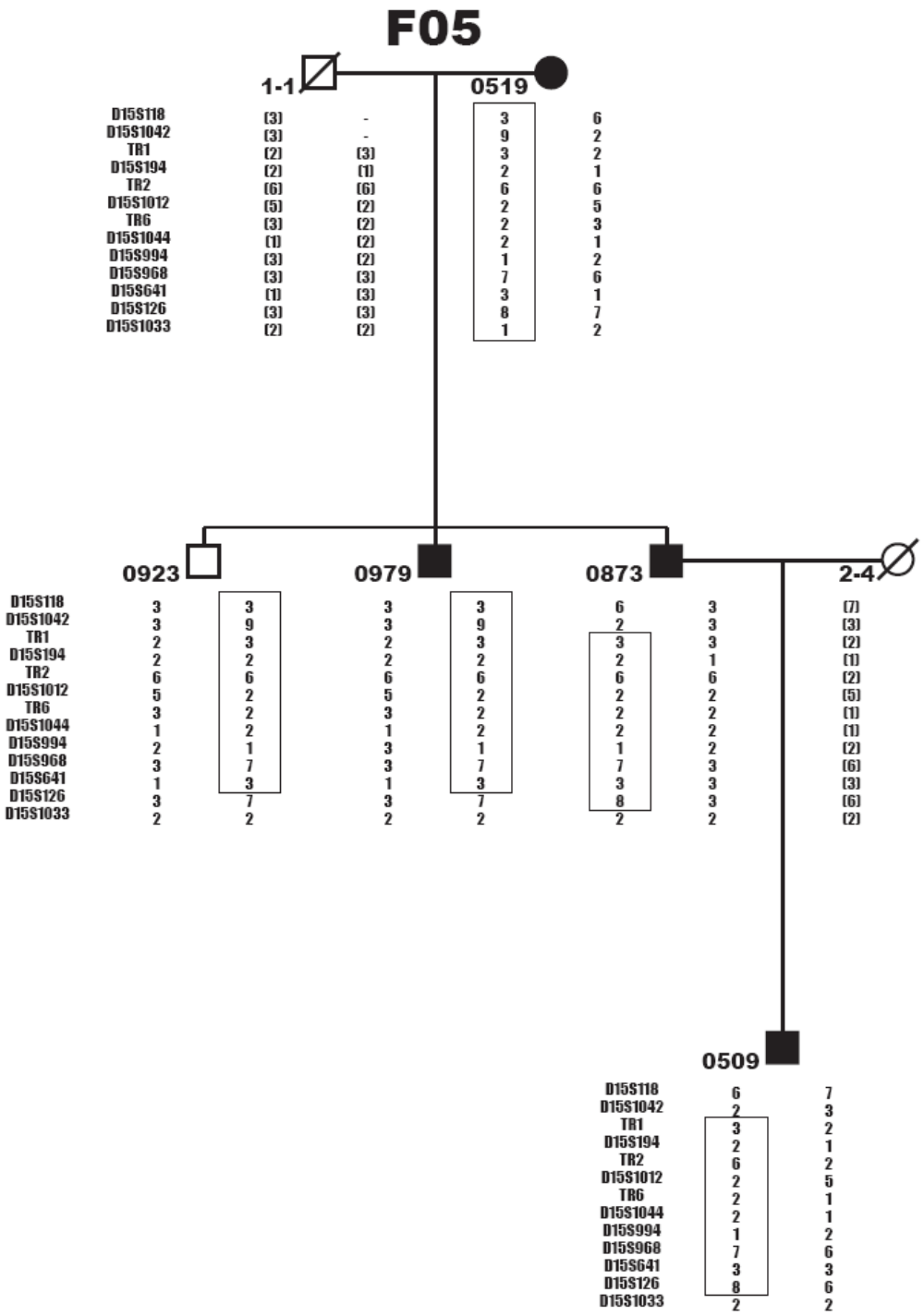


Figure 6. Haplotype analysis of pedigree F5. Black square or circle represents the affected person, open square or circle represent unaffected person. The square represents a male member and the circle represents the female member of the family. The numbers represent the alleles and the box represents the linked haplotype block. The diagonal line across the square or circle represent the ceased person.

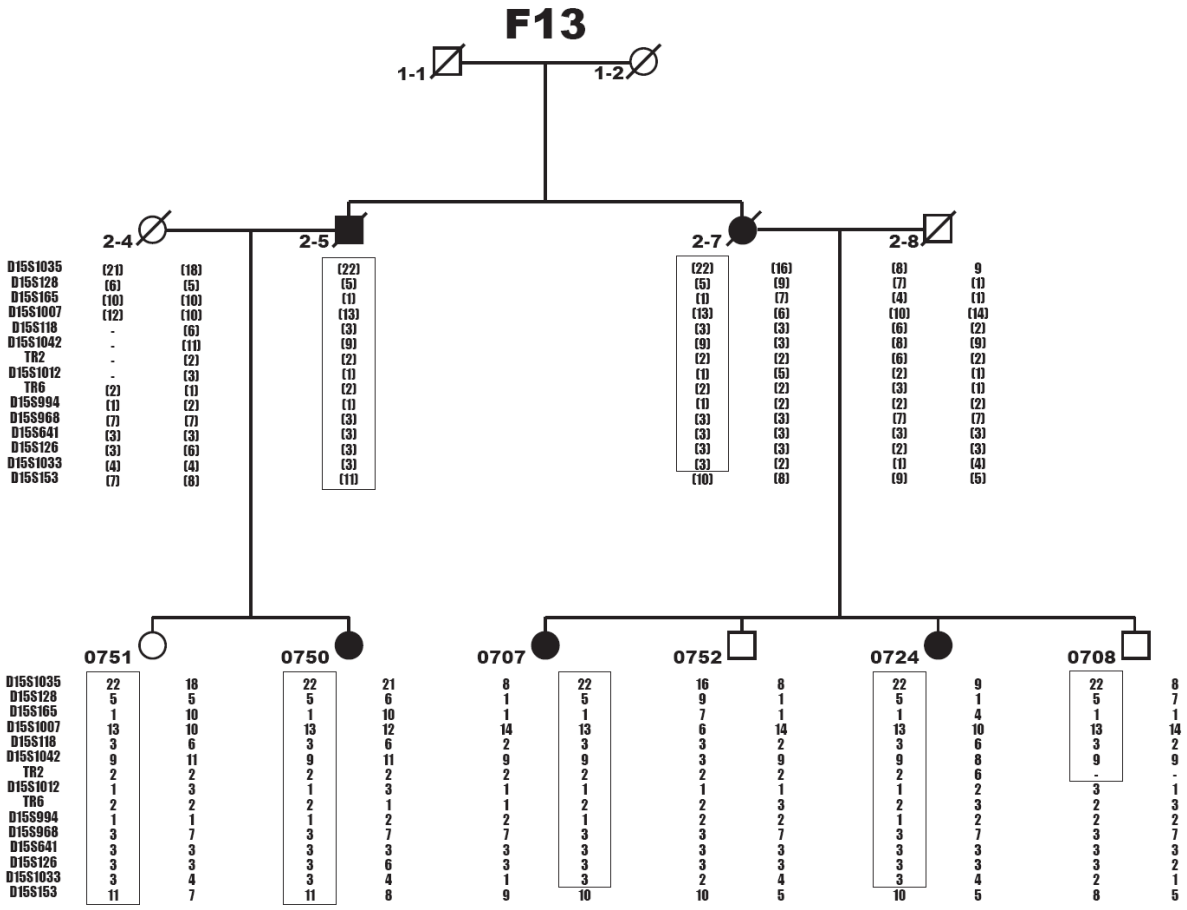


Figure 7. Haplotype analysis of pedigree F13. Black square or circle represents the affected person, open square or circle represent unaffected person. The square represents a male member and the circle represents the female member of the family. The numbers represent the alleles and the box represents the linked haplotype block. The diagonal line across the square or circle represent the ceased person.

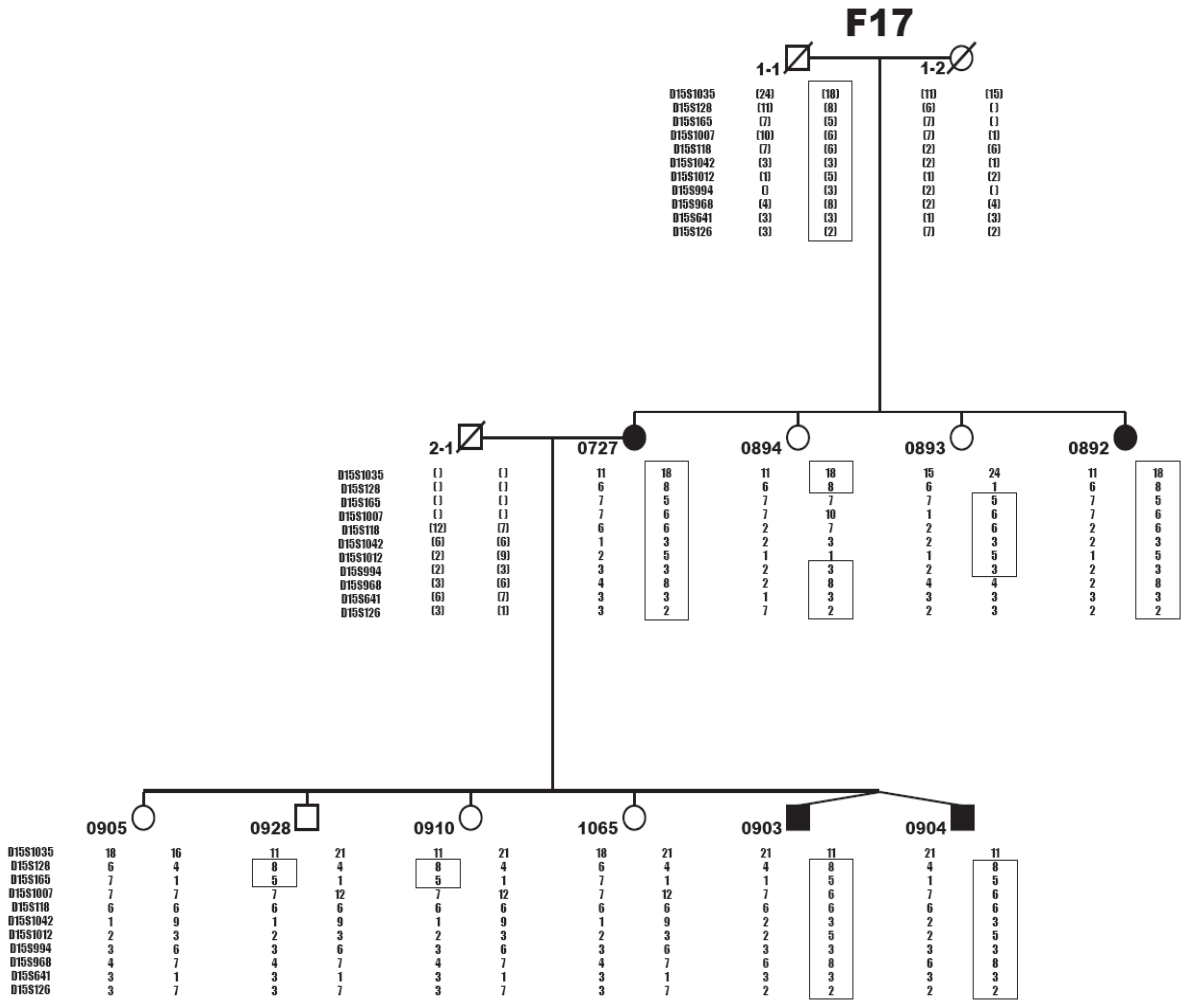


Figure 8. Haplotype analysis of pedigree F17. Black square or circle represents the affected person, open square or circle represent unaffected person. The square represents a male member and the circle represents the female member of the family. The numbers represent the alleles and the box represents the linked haplotype block. The diagonal line across the square or circle represent the ceased person.

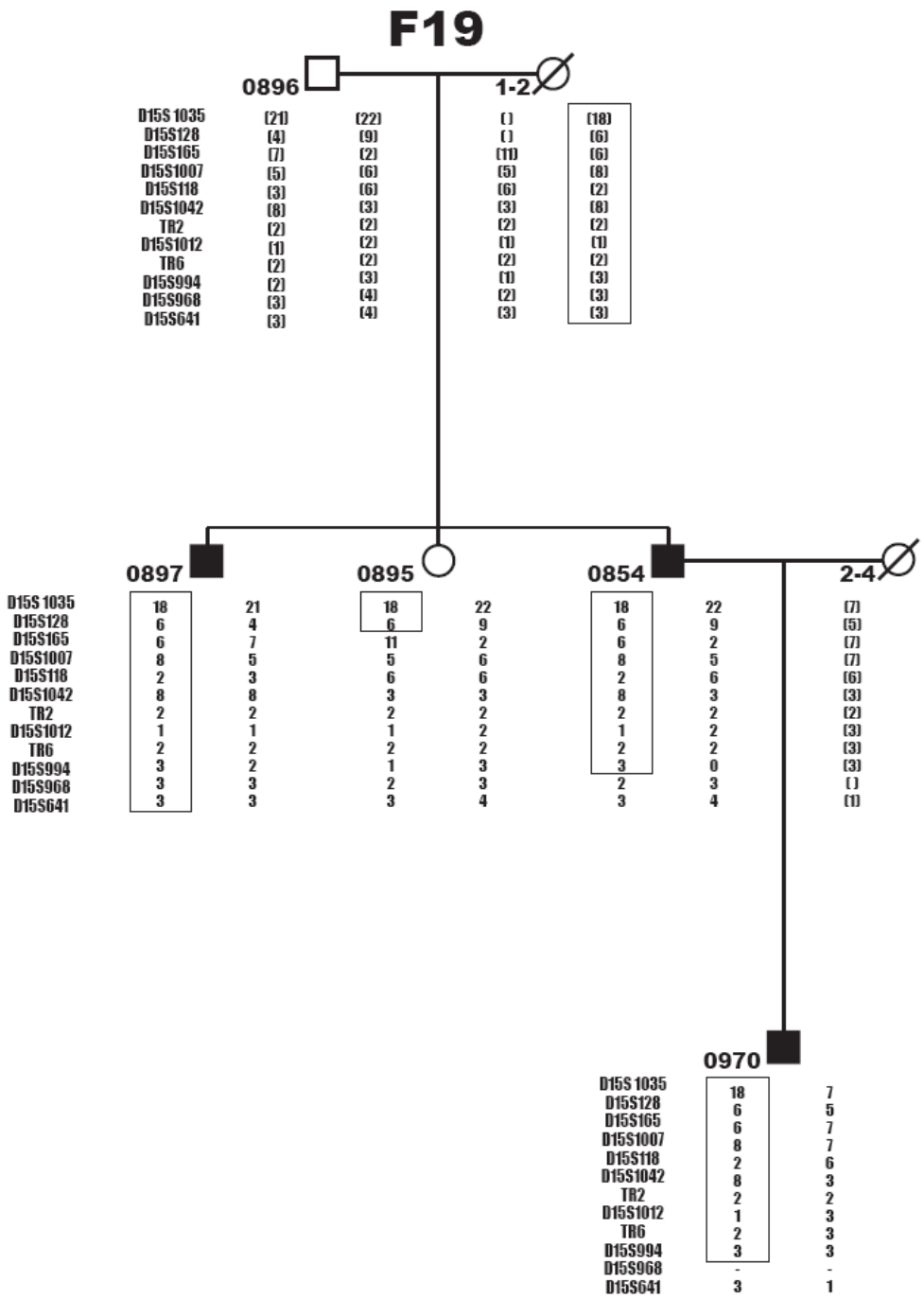


Figure 9. Haplotype analysis of pedigree F19. Black square or circle represents the affected person, open square or circle represent unaffected person. The square represents a male member and the circle represents the female member of the family. The numbers represent the alleles and the box represents the linked haplotype block. The diagonal line across the square or circle represent the ceased person.

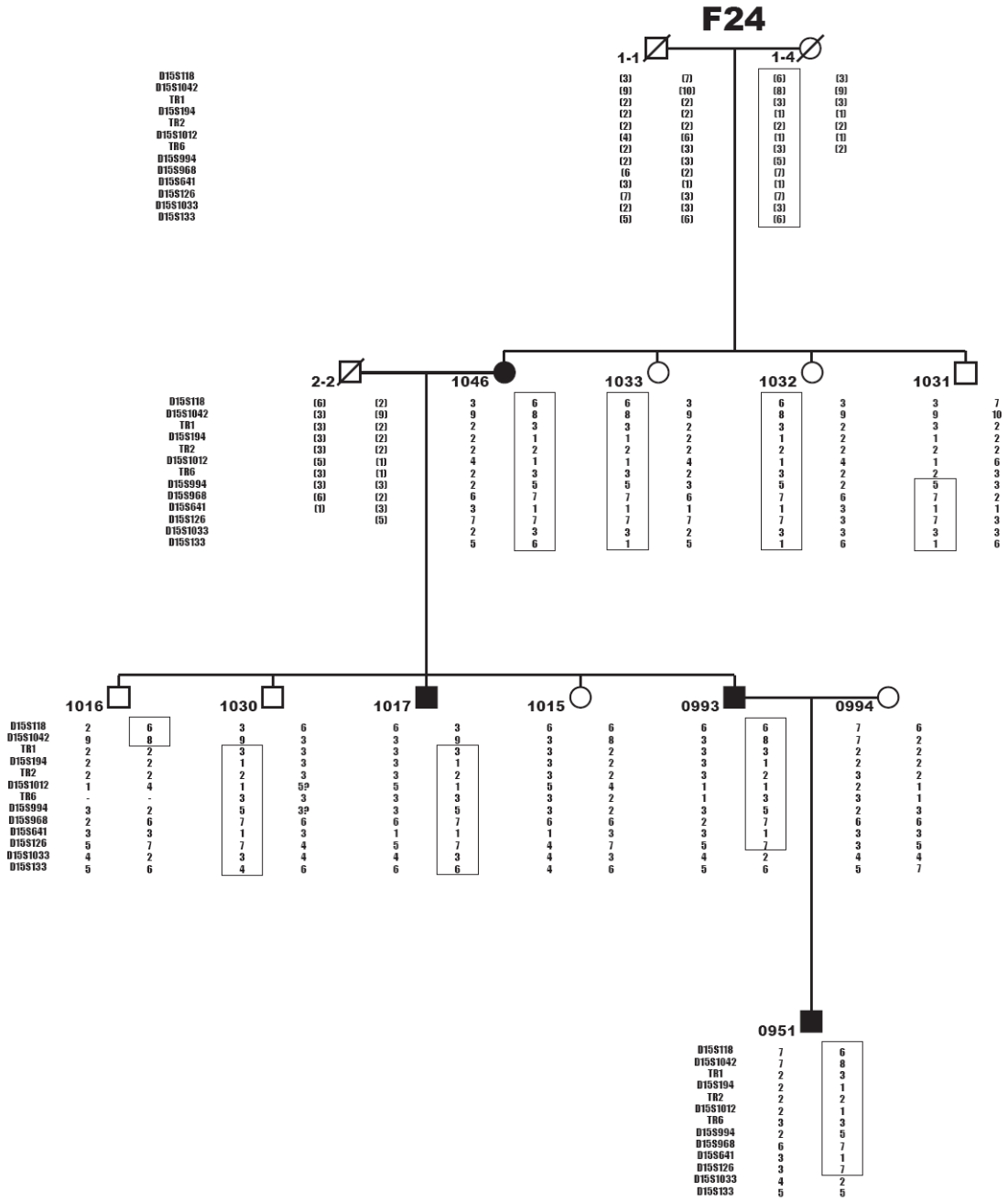


Figure 10. Haplotype analysis of pedigree F24. Black square or circle represents the affected person, open square or circle represent unaffected person. The square represents a male member and the circle represents the female member of the family. The numbers represent the alleles and the box represents the linked haplotype block. The diagonal line across the square or circle represent the ceased person.

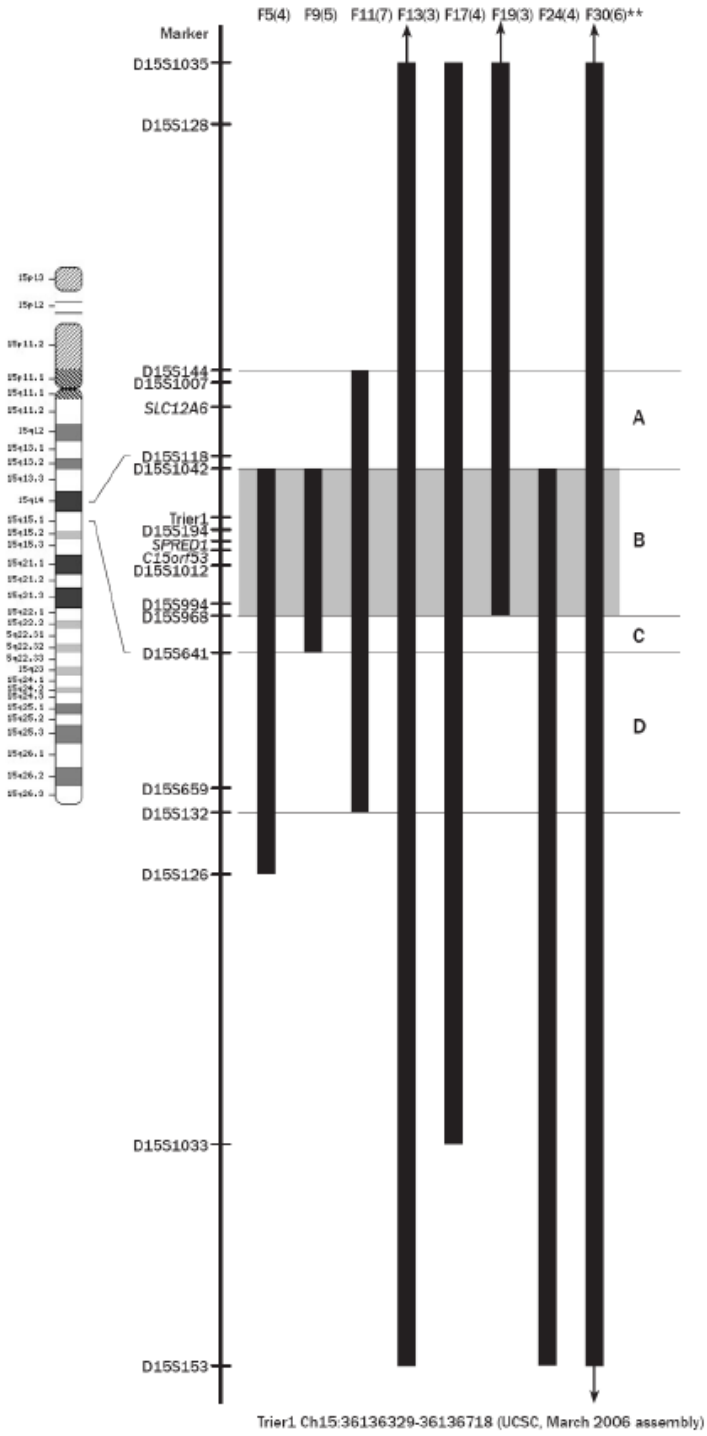


Figure 11. Overlapping intervals of chromosome 15q that segregate with psychosis in German pedigree with periodic catatonia. Black bars represent regions that segregated with disease in each pedigree, and the ends of the bars represent markers that define the boundaries of the linked haplotypes. Arrows indicate that the susceptibility region is extending further to proximal or distal. Numbers of affected pedigree members are given in brackets. Regions A to D indicate sub-regions of the F11 linkage region that are shared by at least four other pedigree. **Data for F30 were taken from the literature (Stöber et al., 2002).

3.2 Non-parametric linkage analysis

3.2.1 Non-parametric linkage analysis comprising 11 pedigrees

Non-parametric linkage analysis comprising all 11 pedigrees was performed in collaboration with Institute of Medical Biometry, Informatics and Epidemiology, University of Bonn. The analyses result is presented in Figure 12.

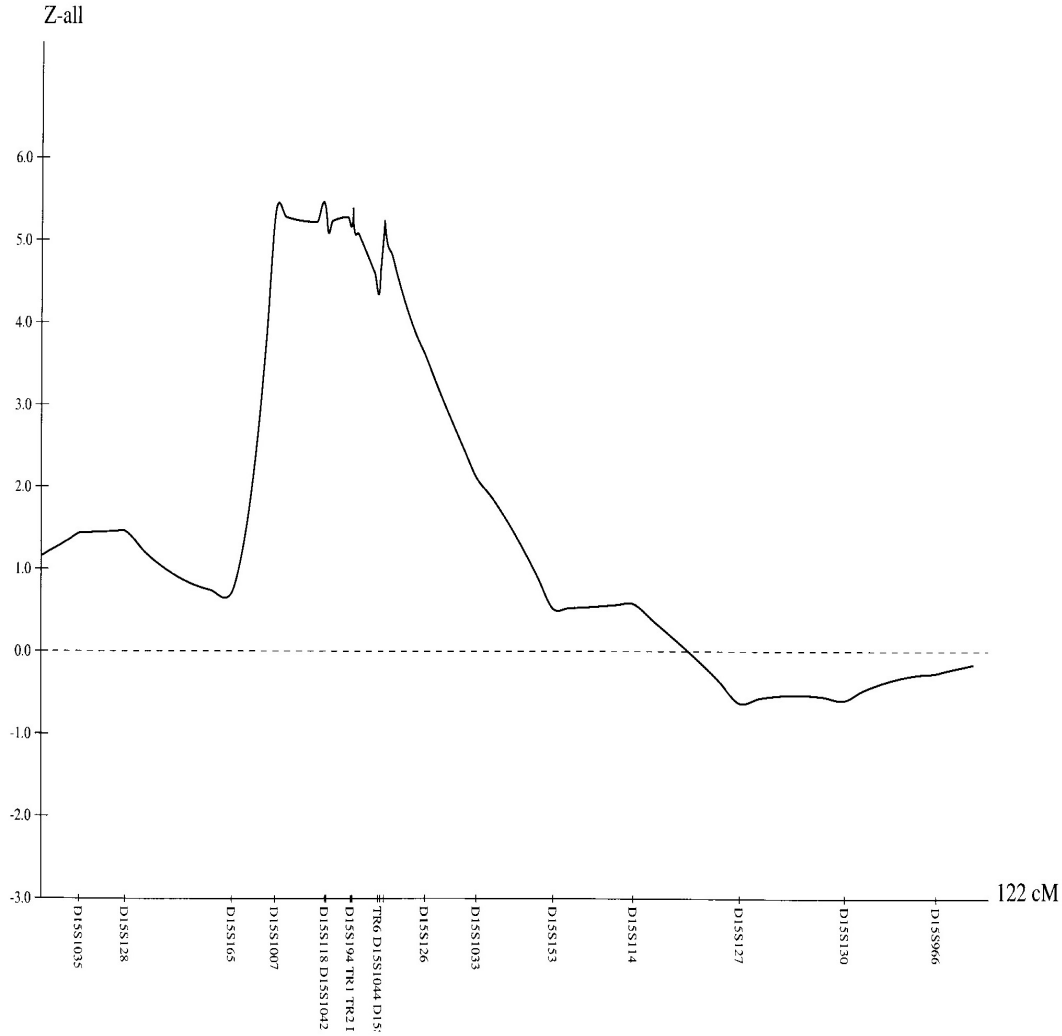


Figure 12. Non-parametric linkage analysis results for eleven pedigrees with SCZD10. The Y-axis represents NPL scores (Z_{all}), and the X-axis represents the position on chromosome 15 (in cM). The NPL_{all} score was above 5.0 for a region of 10.75 cM from 25.90 to 37.47 cM, and from 40.10 to 40.21 cM.

Analysis using 19 markers in the region between D15S1035 and D15S153 revealed a linkage region with two peaks at position 36.01 cM (NPL score 5.39, p = 0.001), and 40.21 cM (NPL score 5.24, p = 0.001). This result confirmed the previous finding of linkage in chromosome 15q and previous fine mapping that was done for pedigrees F11 and F9 by Stöber and colleagues (2002).

3.2.2 Non-parametric linkage analysis for pedigrees linked to chromosome 15q (7 pedigrees).

Further non parametric linkage analysis was performed for chromosome 15-linked pedigrees in order to see whether the NPL_{all} score peak would change, for example move towards one specific marker. Pedigrees included in this calculation were F5, F9, F11, F13, F17, F19 and F24. The analysis was done using SIMWALK2 program through Madeline interface program and presented in Figure 13.

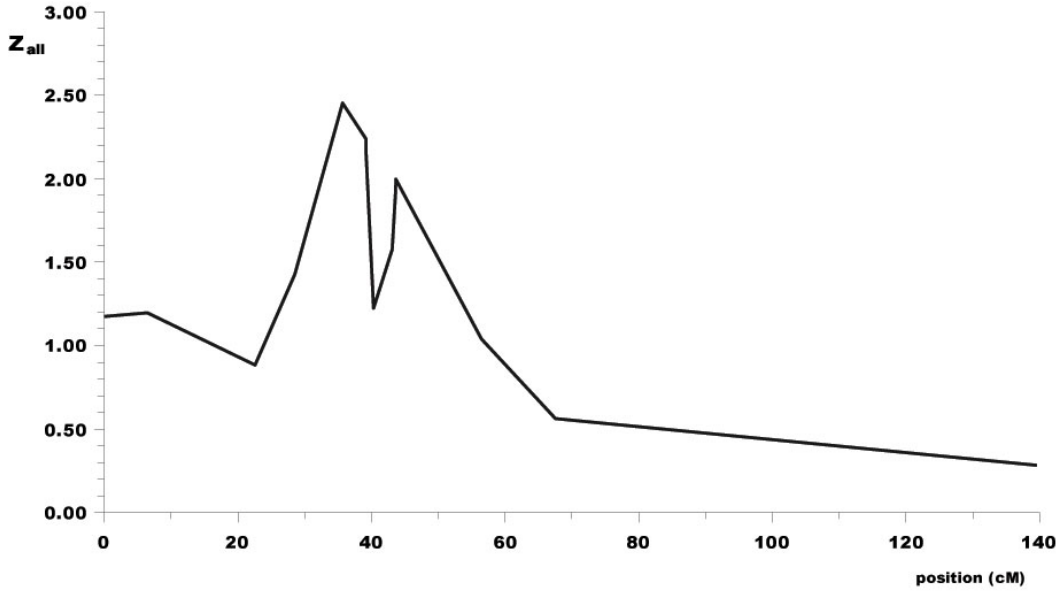


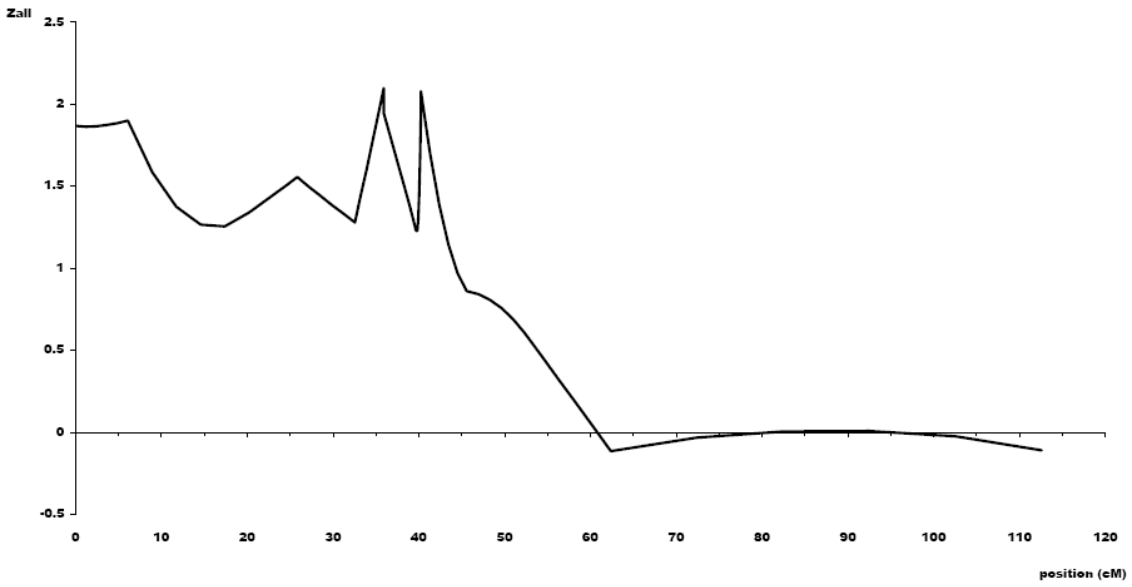
Figure 13. Non-parametric linkage analysis results for seven pedigrees with confirmed linkage to chromosome 15. The peak region with an NPL score > 2.0 was narrowed down to ~3.5 cM.

As shown in Figure 13, there was indeed a narrower susceptibility region, with a peak at marker D15S118 (NPL_{all} score 2.45, sub-region A), and a second peak at marker D15S994 (NPL_{all} score 1.99, sub-region B). Susceptibility regions defined by this calculation were located between markers D15S1042 and D15S1012 (sub-region B), and between markers D15S968 and D15S994 (sub-region B). Statistical power was reduced as expected when using a smaller number of pedigrees. However, the narrower linked region was in accordance with the fine mapping results shown in Figure 11, and may prove helpful to select candidate genes for further analysis. The highest peak at marker D15S118 also supported simultaneous findings from our group for a functional rare promoter variant of a gene located near this marker, i.e. *SLC12A6* (Moser et al., 2008). The variant co-segregates with the disorder exclusively in F11 (Meyer et al., 2005). However, this functional rare variant was only found in pedigree F11 but not in others. Hence, it is interesting to elucidate whether omitting pedigree F11 from the calculation would change the shape and maximum of the peak.

3.2.3 Non-parametric linkage analysis from 6 pedigrees

Having omitted pedigree F11 from the calculation, non-parametric linkage analysis from 6 pedigrees (F5, F9, F13, F17, F19 and F24), as shown in Figure 14(A), revealed that the first peak shifted towards marker D15S194 (sub-region B), while the second peak in marker D15S994 (sub-region B) remained stable. It important to note that non-parametric linkage analysis of pedigree F11 only gave one peak at the region of marker D15S118 (Figure 14B), in accordance with the findings of *SLC12A6* rare variants for this pedigree. Therefore, searching of the responsible DNA variant(s) for PC in other pedigrees will be concentrated on the region close to D15S194 and D15S994.

A.



B.

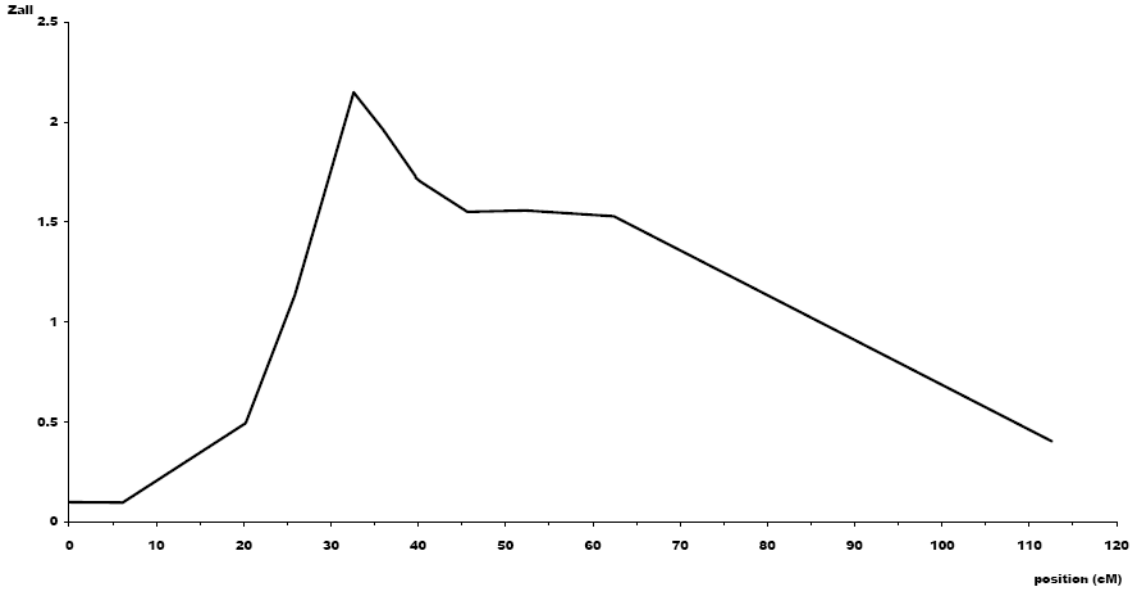


Figure14. Non-parametric linkage analyses result of chromosome 15-linked pedigrees without pedigree F11 (A) and of pedigree F11 only (B). Pedigree F11 gave only one peak in marker D15S118 while the other pedigrees gave two peaks in marker D15S194 and D15S994.

3.3 Catatonic schizophrenia candidate genes in the linkage region

Having narrowed down the susceptibility region, we listed candidate genes that reside in the 15q14-15.1 SCZD10 susceptibility region as shown in Tables 3 and 4. (Order of genesfrom proximal to distal is presented according to that provided by the UCSC map, freeze 2006).

Gene	Description	Analysis	Sub-region
ENSG00000210354	scRNA (or pseudogene)		B
ENSG00000210360	snoRNA (or pseudogene)		B
C15orf41	Chromosome 15 open reading frame 41 (hypothetical)		B
MEIS2	Homeobox protein Meis2 isoform d		B
ENSG00000206676	Misc_RNA (or pseudogene)		B
TMCO5	Hypothetical protein FLJ35807.		B
SPRED1	Sprouty-related, EVH1 domain containing 1	Excluded	B
FAM98B	Pedigree with sequence similarity 98, member B (hypothetical)		B
ENSG00000201509	Misc-RNA (or pseudogene)		B
RASGRP1	RAS guanyl releasing protein 1 (calcium and DAG-regulated)		B
C15orf53	**Chromosome 15 open reading frame 53 (hypothetical)**		**B**
ENSG00000210398	snoRNA (or pseudogene)		B
FLJ39531	Hypothetical gene		B
THBS1	Thrombospondin 1	a	B
FSIP1	Fibrous sheath interacting protein 1		B
GPR176	G protein-coupled receptor 176	a	B
ENSG00000210423	snoRNA (or pseudogene)		B
EIF2AK4	Eukaryotic translation initiation factor 2 alpha kinase 4	**Excluded** (unpublished data)	B
ENSG00000210437	Mt-tRNA (or pseudogene)		B
ENSG00000210449	scRNA (or pseudogene)		B
SRP14	Signal recognition particle 14 kDa (homologous Alu-RNA binding protein)	a	B
BMF	Bcl2-modifying factor	a	B
BUB1B	BUB1 budding uninhibited by benzimidazoles 1 homolog beta (yeast)	**Excluded** (unpublished data)	B
PAK6	p21(CDKN1A)-activated kinase 6	a	B
FLJ38596	Hypothetical gene		B
PLCB2	Phospholipase C, beta 2	**Excluded** (unpublished data)	B,C
FLJ43339	Hypothetical protein DKFZp686N1468		B,C

Table 3. SCZD10 positional candidate genes and transcribed elements located in the 15q14-15.1 locus sub-regions B and C.
* Analysis is in progress

Gene	Description	Analysis
DISP2	Dispatched B	
C15orf23	Chromosome 15 open reading frame 23	
IVD	Isovaleryl Coenzyme A dehydrogenase	
BAHD1	Bromo adjacent homology domain containing 1	
D4ST1	Dermatan 4 sulfotransferase 1	
AK098781	Hypothetical protein FLJ25915	
CCDC32	Hypothetical protein MGC20481	
RPUSD2	RNA pseudouridylate synthase domain containing	
CASC5	Cancer susceptibility candidate 5 isoform 1	
RAD51	RAD51 homolog protein isoform 1	
FAM82C	Pedigree with sequence similarity 82, member C	
GCHFR	GTP cyclohydrolase I feedback regulatory	
DNAJC17	DnaJ (Hsp40) homolog, subpedigree C, member 17	
ZFYVE19	Zinc finger, FYVE domain containing 19	
PPP1R14D	Protein phosphatase 1, regulatory (inhibitor) subunit 14D	
SPINT1	Hepatocyte growth factor activator inhibitor 1	
RHOV	Ras homolog gene pedigree, member V	
VPS18	Vacuolar protein sorting 18	Excluded (Ekici et al., 2004)
DLL4	Delta-like 4 protein precursor	Excluded (McKeane et al., 2005)
CHAC1	Hypothetical protein LOC79094	
INOC1	Hypothetical protein DKFZp686N224	
EXDL1	Hypothetical protein LOC161829	
CHP	Calcium binding protein P22	Excluded (Ekici et al., 2004)
OIP5	Opa interacting protein 5	
NUSAP1	Nucleolar and spindle associated protein 1	
NDUFAF1	NADH dehydrogenase (ubiquinone) 1 alpha	
RTF1	Paf1/RNA polymerase II complex component	
ITPKA	1D-myo-inositol-trisphosphate 3-kinase A	Excluded (Ekici et al., 2004)
LTK	Leukocyte tyrosine kinase isoform 1	

Table 4. SCZD10 positional candidate genes located in the 15q14-15.1 locus sub-region C. [a]Not yet analyzed

Theoretically, all of the genes or functional elements in this region are candidates for susceptibility to periodic catatonia. In Tables 3 and 4, all candidate genes are ordered according to their position on the chromosome. Genes that reside in sub-region B, and are exclusively expressed in the brain, represent most interesting candidates for mutational analysis.

3.4 Mutation analysis for SPRED1

The *SPRED1* transcript, consisting of 8 exons, is 3829 bps in length (Ensembl). Primer pairs were designed for exons (including 20-50 bp exon-intron junction) and predicted promoter region using Primer3 software (Rozen et al., 2000). Promoter prediction was done using PROSCAN 1.7 program online (http://thr.cit.nih.gov/molbio/proscan/).

Direct sequencing analysis was performed for sample number 744, 834 and 568. The sequencing results are then aligned against reference sequence from database (UCSC, May 2004 freeze) using Clustalw program available from European Bioinformatics Institute (www.ebi.ac.uk) and summerized in Table 5.

SNP	Alleles	Genotype Frequency		Result	
rs7182		G/G		568	A/A
	G/A	G/A	0.292	744	A/A
445		A/A	0.708	834	A/A
rs7180		C/C		568	A/A
	C/A	C/A	0.292	744	A/A
446		A/A	0.708	834	A/A
rs3751		T/T		568	C/C
	G/A	C/T	0.261	744	C/C
526		C/C	0.739	834	C/C

Table 5. Direct-sequencing results of *SPRED1*. Sample 744 and 834 are from pedigree F11, sample 568 is from pedigree F9. Genotype frequency was based on data from European American descents population (taken from Ensembl Human SNPView database).

We found already described SNPs in exon 4 (rs7182445), intron 5 (rs7180446) and exon 8 (rs3751526). All three patients had a similar haplotype box for these SNPs. However, the two exonic SNPs are non-synonymous, and all three SNPs are common variants for European-American descent population. We also found the C variants of two SNPs, one that creates a putative methylation site in the predicted promoter area (rs1522781), and one in intron 1 (rs1522782) in all three affected subjects. These SNPs were also found in all pedigree members of F9 that shared the susceptibility haplotype block. At present, there are

no available data about the population frequency for these SNPs. However, one pedigree member lacking the susceptibility haplotype block also had the C variants of these SNPs, and so the unrelated control. Hence, *SPRED1* might not be responsible for catatonic schizophrenia in our pedigrees.

3.5 Ultraconserved element

Six ultra-conserved elements are located in the region between markers D15S1042 and D15S659, the broader periodic catatonia susceptibility interval defined by Stöber et al. (2002), as shown in Table 6. Direct sequencing analysis was performed for three DNA samples, namely sample number 744 and 834 (from F11), and 568 (from F9). The sequencing results from these samples are then aligned against reference sequence from database (UCSC, May 2004 freeze).

ultra conserved element				within
Name	**type**	**length**	**position**	**gene**
uc.382	non exonic	200	chr15:33634968-33635167	no
uc.383	non exonic	269	chr15:34535983-34536251	no
uc.384	possibly exonic	266	chr15:34681449-34681714	HH114
uc.385	possibly exonic	209	chr15:34901726-34901934	MEIS2
uc.386	non exonic	203	chr15:35238065-35238267	no
uc.387	exonic	238	chr15:39748110-39748347	AL713737

Table 6. Six ultra conserved (uc) elements at 15q15 between D15S1042 and D15S659 (sub-regions B to D), according to UCSC (May 2004 freeze) and Bejerano et al. (2004). Three of these ultra conserved elements reside within genes, and the remaining in the intergenic regions.

We found no sequence differences between our samples and the database, hence concluded that these ultra-conserved elements are not susceptibility factors for SCZD10 in our pedigree.

3.6. Haplotype analysis on 1q42 and 6q loci

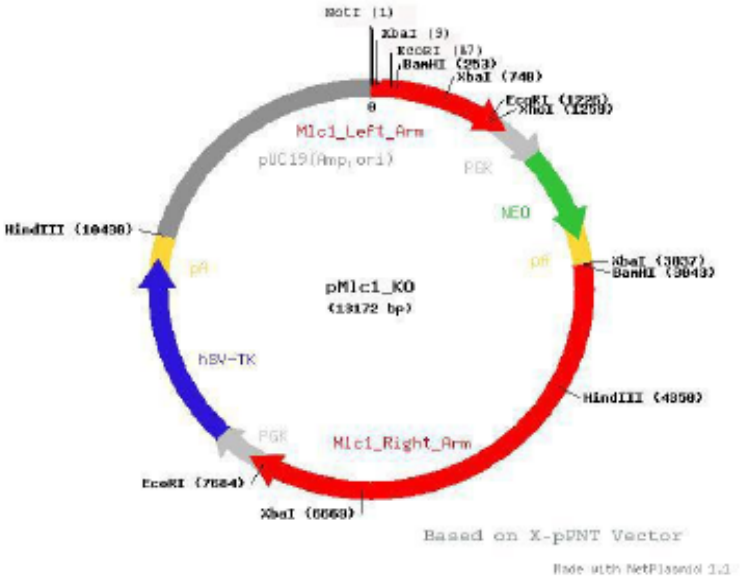
The additional haplotype analysis result on 1q42 and 6q loci revealed that marker D1S419 (1q42) was segregating in affected members of F17, F19 and F24 only. Markers D6S1715, D6S292 and D6S311 (6q) segregated in F5 and F19. Furthermore, only one pedigree that was not linked to chromosome 15 (F15) showed segregation for the 6q locus. Thus, these results further confirmed the strong linkage to the 15q14-15.1 region.

The second sub-study focused on investigation of periodic catatonia locus on chromosome 22q13.33. Meyer and colleagues (2001) described a rare polymorphism, Leu309Met, in the *MLC1* gene. The methionine-encoding variant, which was not present in the controls, was found co-segregating with the syndrome in the large pedigree that supports the 22q13.33 locus. Preliminary steps from our group towards elucidating the function of *MLC1* taken in this study were the construction of mouse knockout vector for *MLC1* and the yeast-two hybrid approach for finding the protein binding partner. The results presented below are of 1) preliminary steps of *MLC1* function elucidation, 2) the fine mapping results of periodic catatonia locus in the pedigree 20 and 21 that mapped to 22q13.33, and 3) sequencing analysis of one SNP in *BRD1* to prove that *MLC1* could not be excluded as periodic catatonia candidate gene.

3.7. *MLC1* knockout vector (pMLC1-KO)

We have constructed a mouse knockout vector for mouse *Mlc1* based on XpPNT vector. The final MLC1 knockout vector construct (pMLC1-KO) map is depicted in Figure 16(A). When quality controlling by sequencing and restriction analysis, we found an inconsistency with the predicted sequence according to UCSC database (March 2006 Freeze). The sequence analysis of pMLC1-KO revealed that there was a deletion of about 0.5 kb in size at the right arm containing the intron 3 and exon 4 of mouse *Mlc1*,as presented in Figure 16(B), but we presumed that this deletion will not interfere with the recombination event.

A.



B.

```
Sac1
gagctccttccgtatccggtagattgacaatgacgctgtgcctgtgtg                primer Xba_For
Gttccgattattgtagattgacaacgatgatgtgcctgtgtggttccgattattgtagat     (#63)
tgacaacgatgctgtgcctgtgtggtcccgattattgtagattgacaacgatgatgtgcc
tgtgtggtcccgattattgtagattgacaacgatgctgtgcctgtgtggttccgattatt
gtagattgacaacgatgctgtgcctgtgtggtcccgattattgtagattgacaacgatgc
            KpnI                                        KpnI
tgtgcctgtgtggtaccgattattgtagattgacaacgatgctgtgcctgtgtggtaccg
attattgtagattgacaacgatggtgtgcctgtgtggtcccgattattgtagattgacaa
cgatgctgtgcctgtgtggtcccgattattgtagattgacaacgatgctgtgcctgtgtg
KpnI                                        KpnI
gtaccgattattgtagattgacaacgatgctgtgcctgtgtggtaccgattattgtagat
tgacaacgatgctgtgcctgtgtggtcccgattattgtagattgacaacgatgttgtgcc
tgtgtggtcccgattattgtagattgacaacgatgctgtgcctgtgtggtcccgattatt
gtagattgacaacgatgctgtgcctgtgtggttccgattattgtagattgacaacgatgc
tgtgcctgtgtggtcccgattattgtagattgacaacgatgatgtgcctgtgtggtcccg
attattgtagattgacaacgatgctgtgcctgtgtggtcccgattattgtagattgacaa
cgatgctgtgcctgtgtggttccgattattgtagattgacaacgatgctgtgcctgtgtg
gttccgattattgtagattgacaacgatgctgtgcctgtgtggttccgattattgtagat
tgacaacgatgctgtgcctgtgtggtcccgattattgtagattgacaacgatgctgtgcc
                        HindIII
tgtgtgggtcagattattgtagaagcttgaacattgcaacacatgattgtcttagtttta
tgctgagctgagttaaaactcaaacgtcttcagcttggtaggcaaaagctctaaccctga
gctgcacctggcccgcaatgcatggtttcagggttagcatcatgtccaggacacaagact
                        BsaI
caggtgaactcttggctcacttggttctggtctctttacagTGCATCCCCTCAGCAATT   Exon 4
GTGAGCTTCGCTGTAGGGAGGAGAAATGTCAGTGCGgtaagtgaagccttcggagcagcc
```

Figure 16. Knockout vector for *MLC1*. (A) pMLC1-KO construct (B) The 0.5 kb deletion found in the right arm insert. The blue highlights represents flanking regions of the deleted sequences and showed repeated sequences, presumably causing the rearrangement and deletion.

3.8. Yeast two-hybrid

In the initial screening optimization procedures using the MLC1 bait, some transformants were detected. Unfortunately, the real high-throughput screening revealed very low signals, suggesting that the transformants were only artifacts. This result was not so surprising, considering the presence of the full 8 transmembrane domains that prevent the bait from entering the nucleus, where the binding reaction occurs. However, there were successful screenings using baits with 4-5 transmembrane domains (RZPD, personal communication, 2005). This data completed our collaborator's results (unpublished), in which the positive clones could not be found when using the N-terminal or C-terminal domains of MLC1 as baits. With the advanced development of yeast two-hybrid systems nowadays, it is very likely that finding the binding partner of MLC1 with yeast two-hybrid method be possible with the use of the split-ubiquitin system (e.g DUALmembrane system or DUALhunter system from DualSystem Biotech, Switzerland). The split-ubiquitin system will allow the bait and prey to interact outside the nucleus, and thus valuable in finding membrane protein's binding partner(s).

3.9. Fine mapping of pedigree F20 and F21 to chromosome 22q13 and *MLC1*

Meyer and colleagues (2001) described a rare polymorphism, Leu309Met, in the *MLC1* gene. The methionine-encoding variant was found co-segregating with the syndrome in the large pedigree that supports the 22q13.33 locus. Later, Rubie and colleagues (2003) reported another family (pedigree F20) found with Leu309Met allele and SCZD10, but argued that the Leu309Met allele was found in the healthy person. Therefore, the group

excluded *MLC1* as a candidate gene for SCZD10 and stated that pedigree F20 was not useful for fine-mapping previously reported SCZD10 locus at chromosome 22q13.33.

We have then investigated pedigrees F20 and F21 further in detail. Detail haplotype analysis was done for pedigree F21, the first pedigree with L309M mutation in *MLC1*, to find the borders of the mapped chromosome 22q13.33 to periodic catatonia (Figure 17).

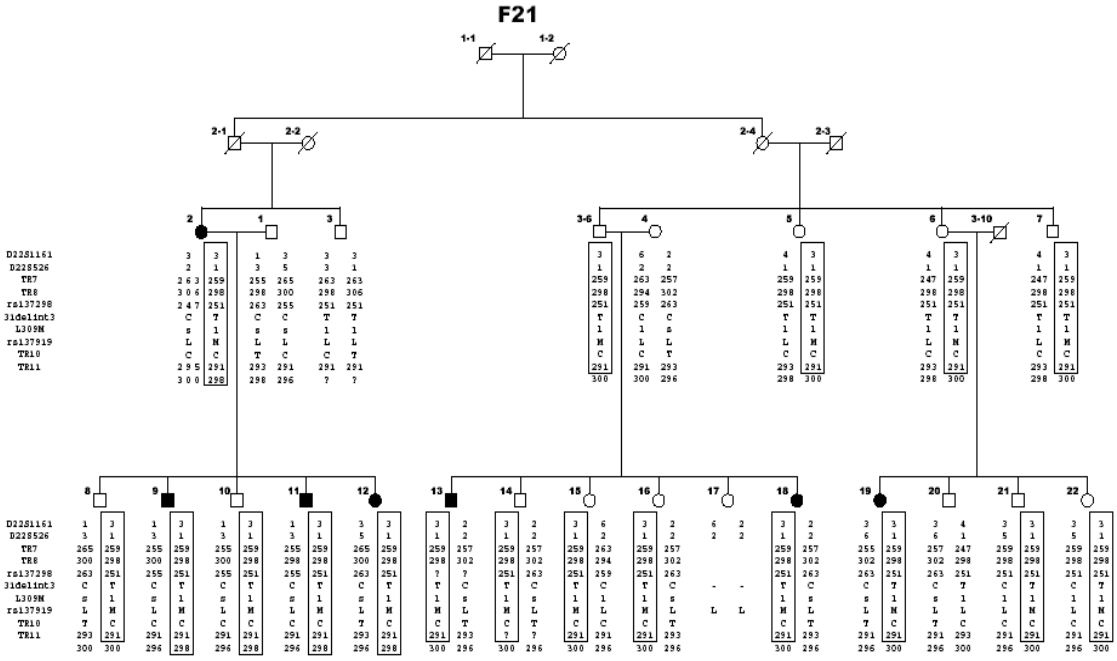


Figure 17. Fine mapping of pedigree 21 with the co-segregating *MLC1* L309M mutation with periodic catatonia.

Haplotype analysis of the pedigree ever found after pedigree 21 with exactly the same L309M mutation, albeit with controversion, namely pedigree F20, revealed that the "healthy" father carries the mutation (Figure 18). Fine mapping of chromosome 22q13.33 markers revealed that he, as his affected son, shares a common haplotype with F21 (Figure 17). Supposed, that the schizophrenia subtype is transmitted by him, and not by the mother of the index patient, recombination events would place the disease-causing gene to a telomeric ~577 Kb interval, a relatively small region. In contrast to Rubie and colleagues

(2002), the haplotype found in F20 is useful to reduce the size of the region of interest considerably. Only 20 genes are located in this interval. Others, including *ARSA*, the gene causing metachromatic leukodystrophy (OMIM #250100), are excluded by this effort.

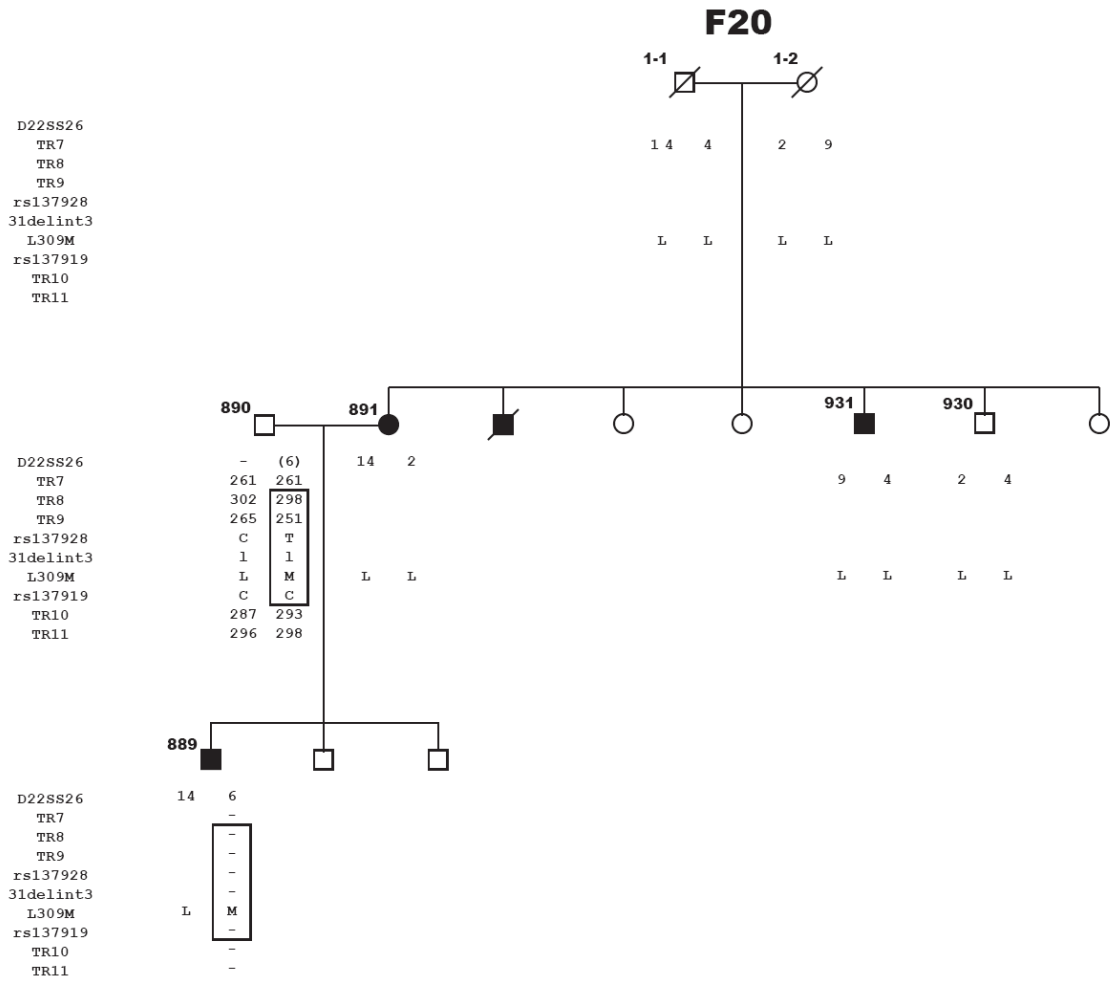


Figure 18. Haplotype analysis of the second pedigree (F20) with L309M mutation in *MLC1*. The fine-mapped region defined from this pedigree is between chr15:48712248-48712545 (UCSC May 2004 freeze) and rs137919.

3.10 Exclusion of *BRD1* as periodic catatonia candidate gene.

Severinson and colleague (2006) previously reported association between microsatellite markers located at 22q13.31-qtel for both schizophrenia and bipolar disorder in a Faeroese sample. The group later found an association of rs138880 C-allele, an SNP located in the promoter of *BRD1*, for schizophrenia, bipolar disorder and the combined group and therefore concluded that *BRD1* was the candidate gene. There was no association of *MLC1* SNPs found in this Faeroese sample for mental disorders despite the linkage to chromosome 22q13.

We have investigated rs138880 in our pedigrees F20 and F21 that showed linkage to chromosome 22q13.33. However, sequencing analysis results revealed an A-allele for rs138880 for person ID 18 and 19 (affected person from pedigree F21) and person ID 890 (from pedigree F20) and therefore excludes BRD1 as a candidate gene for periodic catatonia in pedigrees F20 and F21.

3.11 Recapitulation of mutations in MLC1

Recapitulation of all published *MLC1* mutations (Leegwater et al., 2001, 2002; Tsujino et al., 2003; BenZeev et al., 2002; Patrono et al., 2003), either found in MLC or SCDZ10, known to-date and presentation of their positions in the predicted domains of MLC1 is depicted in Figure 19. Transmembrane domains were predicted using web site-based computer program SOSUI. The mutations found in relation to periodic catatonia were never found in MLC patients, suggesting a different underlying functional effect of the mutations.

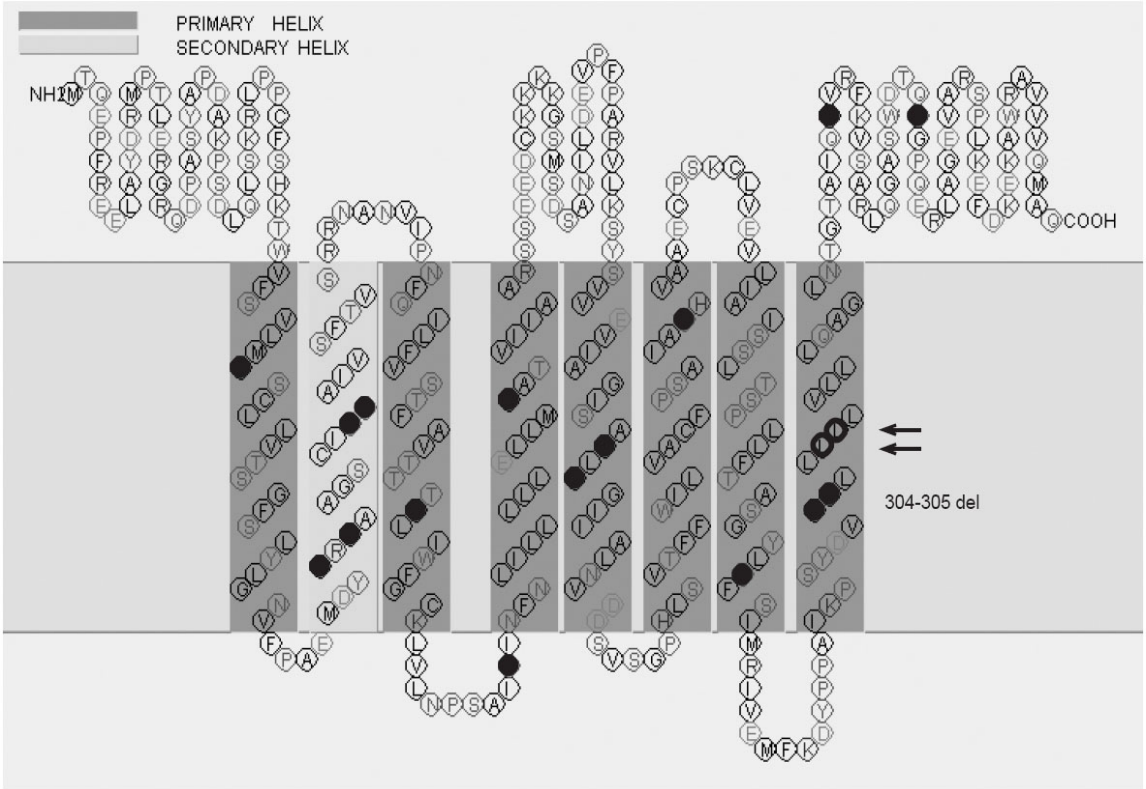


Figure19. Schematic diagram of mutations found for MLC and psychiatric disease in the MLC1 protein. Black-filled circles represent missense and in frame deletion mutations found in MLC, open circles and arrows represent missense mutations found in psychiatric diseases.

Comparative analysis on MLC1 from human, dog, chicken, mouse, crab-eating monkey, frog and zebrafish is depicted in Figure 20. The results also revealed that the amino acid in position 309 found in dog, chicken, frog and zebrafish is methionine like in schizophrenic patients, while the mouse and crab-eating monkey have leucine instead just like in healthy humans. Thus, Leu309Met may represent an atavistic back mutation that is not appropriate in humans with respect to function anymore.

A.

```
human   1   MTQE--PFREELAYDRMPTLER---------GRQ-----DPASYAPDAKPSDLQLSKR---LPPCFSHKTWVFSVLMGSC  61
mouse   1   MTREG-QFREELGYDRMPTLER---------GRQDAGRQDPGSYTPDSKPKDLQLSKR---LPPCFSYKTWVFSVLMGSC  67
rat     1   MTREG-QFREELGYDRMPTLER---------GRQDAGRQDTGSYTPDTKPKDLQLTKR---LPPCFSYKTWVFSVLMGSC  67
chicken 1   MTREE-GYREPFSYDRMPTLER---------GKQ-----ENGNYIPDILSSDLQLSKR---LHPCFIYRTWIFSLLMGSC  62
fugu    1   --------REPFTYSHMPMLER--NGGTLGRRGERVYRPERDSYTVDVRASDLQLDHPEPLKHPCFSCRAWLYSVLIGGS  70
trout   1   MQRDDLSAMEEFSYTQMPTLERGVGGGTVGRRG-CAERERPESYTVDVRASDLQLAHSEKPMHPCLSYRTWLYSVLIGSS  79

                I                   II                  III
human   62  LLVTSGFSLYLGNVFPAEMDYLRCAAGSCIFSAIVSFTVSRRNANVIPNPQILFVSTFAVTTTCLIWFGCKLVLNPSAIN 141
mouse   68  LLVTSGFSLYLGNVFPSEMDYLRCAAGSCIFSAIVSFAVGRRNVSAIPNPQILFVSTFAVTTTCLIWFGCKLILNPSAIN 147
rat     68  LLVTSGFSLYLGNVFPSEMDYLRCAAGSCIFSAIVSFAVGRRNVSAIPNPQILFVSTFAVTTTCLIWFGCKLILNPSAIN 147
chicken 63  LLITSGFSLYLGNIFPSEMDYLRCAAGSCIFSAVVSFAIARNKINVIPNPQILFVSTFAVTTTCLIWFGCKLVLNPSAIN 142
fugu    71  LLITSGFSLYLGNVFPVAMDYLRCAAGAGPHAAIASFAIAKNRNVAVSDPQVVYVSTFAVTTTCLVWFGCKLILNPSAVN 150
trout   80  LLVTAGFSLYLGNVFPAAMDYLRCAAGSSVPAAVVSFAIAKNHHVAVSDPQVVFVSTFAVTTTCLAWFGCKLVLSPSAVN 159

                IV                                  V
human   142 INFNLILLLLLELLMAATVIIAARSSEEDCKKKKGSMSDSANILDEVPFPARVLKSYSVVEVIAGISAVLGGIIALNVDD 221
mouse   148 INFNLILLLLLELLMAATVIISARSSEEPCKKKKGSISDGSNILDEVTFPARVLKSYSVVEVIAGVSAVLGGVIALNVEE 227
rat     148 INFNLILLLLLELLMAATVIISARSSEEPCKKKKGSISDGTNILDEVTFPARVLKSYSVVEVIAGVSAVLGGVIALNVEE 227
chicken 143 INFNLILLILLEIFMATTVIISARSTEDCCMRKKNTTYDSAVVLSNVSFPARILKSYSVIEVIIGISSVFGGIIALNMDV 222
fugu    151 INFNLILMILLEVLMASTVIISARSAEDCCCHRKVCVYERPMVVTPAVFPTRVLKAYSVIEVIVGISSVFGGIIALNMDA 230
trout   160 INFNLILLILLEVLMASTVIISARSAEDCCXXXXXXXXXXMVVETPADFPTRLLKGYSVIEVIARISAVFGGIIALNMDV 239

                VI                          VII
human   222 SVSGPHLSVTFFWILVACFPSAIASHVAAECPSKCLVEVLIAISSLTSPLLFTASGYLSFSIMRIVEMFKDYPPAIKPSY 301
mouse   228 AVSGPHLSVTFFWILVACFPSAIASHVAAECPSKCLVEVLIAISSLTSPLLFTASGYLSFSVMRVVEIFKDYQPAIKS-Y 306
rat     228 AVSGPHLSVTFFWILVACFPSAIASHVTAECPSKCLVEVLITISSLTSPLLFTASGYLSFSVMRIVEIFKDYPPDIKS-Y 306
chicken 223 LVSGPYLSVTFFWILVACFPSAIASHVAAEYPSKCLVEVLIAISSVTSPLLFTASGYLSFSIMQVVDIFKNYPPPVKQSY 302
fugu    231 LLPGPYLSVTFFWILVACFPSAIASHVVEEYPNKCLVEVLIAISSVTSPLLFSASGFLSSSVISFIEIFLHNVETSKQSY 310
trout   240 LLPGPYLPVTFFWILFACFPRAIARHVVAEYPNKCLVEMLIAISSVTSPLLFSASGFLSCSVLSFIDIFLHEVETAKQSY 319

                VIII
human   302 DVLLLLLLVLLLQAGLNTGTAIQCVRFKVSARLQGASWDT-QNGPQERLAGEVARSPLKEFDKEKAWRAVVVQMAQ 377
mouse   307 DVLLLLLLLLLLQGGLNTGTAIQCVSFKVSARLQAASWDP-QSCPQERPAREVVRGPLKEFDKEKAWRAVVVQMAQ 382
rat     307 DVLLLLLLLLLLQGGLNTGTAIQCVSFKVSARLQAASWDS-QSCPQERPAGEVVRSPLKEFDKEKAWRAVVVQMAQ 382
chicken 303 DVLLLLMLMLLIQACLTIGTVIQCVNYKTKMKLQDSSWTESQVKKQEYRTTEVSNNTLKDFDKDKAWRAVVVQMAQ 379
fugu    311 DILLLIMVLLLVQAVLTSATVVHCASYKSQLRMGSVDSFDRAHTTKHYCEQISN-GTLQDFDKDRNWRAVMVQMAQ 386
trout   320 EILLLIMALLLVQAVLTLATVVHCASYKSQLRMGGPEWDSLQLPAFHSEQQASNGTLREFDKDKAWRAVVVQMAQ 396
```

B.

```
human     IMRIVEMFKDYPPAIKPSYDVLLLLLLVLLLQAGLNTGTAIQCVRFKVSARLQGASWDT 342
Macaca    VMRIVEMFKDYPPAIKPSYDVLLLLLLVLLLQAGLNTGTAIQCVRFKVSARLQGASWDT 342
mouse     VMRVVEIFKDYPPAIK-SYDVLLLLLLLLLLQGGLNTGTAIQCVSFKVSARLQAASWDP 347
dog       IMRIMEIFKDYPPAIKQSYDVLLLLLMLELLLQAGLNTGTVIQCVSFKL-----GASWDA 349
          :**::*:********* *********:* ****.******.**** **:     .****.
```

Figure20. (A) Alignment of MLC1 sequences from different vertebrates using ClustalW software. The 8 transmembrane domains are boxed and indicated by Roman numerals. Residues identical in at least 5 MLC1 orthologues are in black boxes and residues that are similar are indicated in gray (taken from Boor et al., 2005). (B) Emphasize of human, mouse, monkey and dog MLC1 sequences alignment in the transmembrane domain 8. Box indicates position of Leu309 in human.

Chapter 4

Discussion

In a genome-wide linkage scan, Stöber and colleagues (2000) found evidence for a major susceptibility locus on chromosome 15q15 (maximum nonparametric LOD score of 3.57), and a further potential locus meeting criteria for suggestive evidence for 22q13.33, pointing to genetic heterogeneity in periodic catatonia. In the second genome-wide scan, Stöber et al. (2002) found the overlapping region with the previous genome-wide scan result on chromosome 15q and were able to refine the disease gene locus to an 11-cM region between D15S1042 and D15S659. Meyer and colleagues (2001) described a rare polymorphism, Leu309Met, in the *MLC1* gene. The methionine-encoding variant was found co-segregating with the syndrome in the large pedigree that supports the 22q13.33 locus.

4.1 Fine Mapping schizophrenia susceptibility locus at chromosome 15q14-15.1, and exclusion of major candidate genes

In contrast to Mendelian traits, the expected patterns of genetic variation at the genes underlying complex traits are still obscure. Relatively little is known about the specific genetic variants that underlie these traits (Chakravarti 1999). Zwick and colleagues (2000) intensely discussed the possible models of complex traits. Accordingly, among many possible models, two classes of models make opposite predictions about the nature of genetic variation underlying complex traits. At one extreme, the "*complex trait - rare variant*" model predicts that phenotypic variation in complex traits will be caused by numerous, individually

rare genetic variants at multiple loci (Lander 1996; Collins et al 1997). This pattern is similar to that observed for loci that underlie Mendelian traits. At the other extreme, the "*complex trait - common variant*" model predicts that common disease variants will be few at any particular locus, but be relatively common in natural populations, and be shared across multiple subpopulations. Any complex trait might arise from the interaction of both rare and common genetic variants. In contrast to Mendelian subsets of complex diseases in breast cancer (*BRCA1* and *BRCA2*), colon cancer (*APC*) and heart disease (*LDL* receptor gene), progress on psychiatric disorders has been limited, except maybe for *DISC1*.

For the past decades, the dominant study design for investigation of the genetic basis of inherited diseases has been linkage analysis in families (Carlson et al 2004). Typically, a nonparametric linkage analysis will implicate several genetic regions as targets for further investigation. These regions, often 10 – 20 Mb in size, remain intractably large for effective positional cloning efforts.

Bipolar, schizophrenia, and schizoaffective disorders are common, and highly heritable psychiatric disorders, for which familial co-aggregation, as well as epidemiological and genetic evidence, suggests overlapping etiologies (Taylor and Fink 2003; Fallin et al 2005). Linkage and association studies from *DISC1* lend a strong support for this view (Porteous and Millar 2006). In line with this suggestion, a review from Chagnon (2005) on autism susceptibility loci showed that there is a shared chromosome 15 susceptibility region for autism, ADHD, schizophrenia, periodic catatonia and bipolar disorder at the region between markers ACTC and D15S659.

The two genome-wide scan studies by Stöber and associates (2000 and 2002) were the starting point of this study. In the first genome-wide study, one pedigree showed linkage

to a chromosome 22q locus, whereas the remaining eleven pedigrees gave a signal for chromosome 15. However, it was not clear to what extent these eleven pedigrees map to 15q15. Previous fine mapping results in the second genome-wide scan study put the periodic catatonia gene locus to a region between markers D15S1042 and D15S659 of approximately 11 cM, an interval with ~70 annotated genes. Previous refinements of the chromosome 15 susceptibility locus from Stöber´s and our group were mainly based on two pedigrees (F9 and F11).

Our results reveal seven pedigrees with linkage to 15q, whereas the remaining four pedigrees clearly show exclusion of this locus. We refined the candidate region on 15q15 by defining the linked haplotypes in family F11 (the largest family), and in six other families that show linkage to this region. This delineated four sub-regions (A, B, C and D) from D15S1042 to D15S132, respectively, that all showed linkage in at least three other 15q-linked families studied. Sub-region B (4.3 Mb) is considered the refined map and the most interesting, as seven 15q-linked families share this interval. An important but less interesting area is the small sub-region C (1.2 Mb), supported by six 15q-linked families. This interval contains more genes than sub-region B, among these being certain interesting candidates.

Based on Lander and Kruglyak (1995), our 15q14-15.1 fine mapping derived from seven families does not reach significant criteria (NPL score > 4.8, $p < 0.000022$). However, Lander and Kruglyak's criteria were actually for genome-wide significance based on a dense map and on complete information extraction, which do not apply to our experiment with a fraction of missing genotypes, and may therefore be too stringent. Since unifying criteria for significant and suggestive linkages based on NPL scores have not been described in the literature, Chen and colleagues (2005) defined NPL scores of > 3.0 as significant evidence of

linkage, and scores of > 2.0 as suggestive evidence of linkage on the basis of the nominal empirical *p* values. Others (Kaminen et al. 2003; Laivuori et al. 2003; Friedrichsen et al. 2004; Middleton et al. 2004) have used similar criteria. Statistical power was reduced as expected when we calculated NPL score using fewer families. Nevertheless, our 15q14-15.1 susceptibility locus from seven pedigrees still reached suggestive evidence for linkage according to Chen et al (2005).

Calculation comprising seven 15q-linked pedigrees showed a peak at D15S118, in line with other findings (Freedman et al 2001b; Leonard and Freedman 2006; Williams et al 1999). Recently, our group described a rare variant in the *SLC12A6* promoter that segregates with the disease in one family (F11), and is highly associated with bipolar disorder in a case control study (Meyer et al 2005). Meanwhile, this variant proved to be functional (Moser et al., 2008). This finding is in accordance with the fact that most of the knowledge about the genetics of common diseases relates to rare families segregating high-risk alleles (Botstein and Risch 2003; Meyer et al 2005). Interestingly, F11 is the only family that revealed an NPL peak at marker D15S118 (sub-region A) near *SLC12A6* (Figure 4b). Calculation omitting this family resulted in a shifted NPL peak more telomeric toward D15S194 (sub-region B), the recently refined map. This observation supports the presence of a second susceptibility gene or functional element at the chromosome 15q14 candidate schizophrenia locus for our families, as *SLC12A6* resides outside sub-region B. Therefore, we will further focus on investigating genes exclusively in sub-region B.

Moises recently proposed a theoretical strategy to screen for putative candidate genes for schizophrenia (Moises, 2001). He applied four different "screens" successively to all genes located in chromosomal regions for which confirmed linkage with schizophrenia had

been demonstrated. These screens were in brief: 1) all genes present in all chromosomal regions showing significant linkage with schizophrenia; 2) functional relationship between the selected genes; 3) definition of functional pathways and key members therein, and 4) predictive power of the selected genes to explain major symptoms in schizophrenia. About 90 genes of the total human genome have passed all four screens. Of these, the genes *PLCB2*, *EIF3S1* and *EIF2AK4* encoding the phospholipase C β2-subunit, the translation initiation factor 3 α1-subunit, and the translation initiation factor 2α kinase 4, had been assigned to chromosome 15q14-15 (Lander et al 2001). However, sequencing of *PLCB2* (sub-regions B and C) revealed no novel variants (unpublished data). Furthermore, according to our fine-mapping data, *EIF3S1* is located in sub-region D, outside of the newly defined core linkage region. No novel variants were found for the third candidate gene according to Moises that is located in sub-region B, *EIF2AK4* (formerly *GCN2*), by direct sequencing (unpublished data).

We found the susceptibility region of interest, namely sub-region B, being narrower than previously described by Stöber's group. In contrast, we could show that not all families share sub-regions C and D, which were previously included as susceptibility loci. Moreover, there are only 19 annotated genes (UCSC, March 2006 freeze) present in sub-region B. The refinement excludes many genes, in line with the findings from Stöber's and our group. Among other candidate genes, *DLL4* (sub-region C), *SLC30A4* (sub-region D), *CX36* (sub-region A) were analyzed and excluded in previous studies (McKeane et al 2005; Kury et al 2003; Meyer et al 2002). Ekici et al (2004) found no disease-causing mutation in *ARHV, VPS18, CHP, ITPKA, KIAA0252, KIAA1403, KIAA0770, TYRO3,* and *SNAP23* in the same families. These genes are also located outside of sub-region B.

Our direct-sequencing results for ultra-conserved elements inside the sub-regions B to D between D15S1042 and D15S659, however, showed exclusion. We concluded that ultra-conserved elements in our susceptibility locus are not related to the disease, in contrast to the findings of Schanze et al. (2006, 2007). Since the data was only coming from an abstract of poster publication, it was not clear whether we have investigated the same conserved elements. As there are no functional variants detected in *SPRED1* from our three schizophrenic patient samples, we conclude that this gene might not be involved in SCZD10 susceptibility in our families with linkage to the 15q14-15.1 locus. Based on the apparently dominant pattern of inheritance in our families, our research focuses mainly on rare variants or putative mutations. Hence, we cannot rule out the possibility of the "complex traits - common variants" model for *SPRED1*, and other candidates, and also not variants within introns, as these were not investigated completely. However, very recently Brems and colleagues (2007) reported that germline loss-of-function mutations in *SPRED1* cause a neurofibromatosis 1-like phenotype, a newly identified autosomal dominant human disorder. Therefore, *SPRED1* is more likely not to be the candidate gene for periodic catatonia.

Considering the possible heterogeneity based on positive scores obtained in the previous genome-wide studies, the author further performed haplotype analysis for 6q. Only two pedigrees (F5 and F19) showed markers that segregate with the disease for 6p. In the big multiplex families (F9 and F11), none of these markers segregated with the disease. Therefore, in this study with the German families, the 6q locus for schizophrenia susceptibility region can be ruled out. Thus, the 15q14-15.1 region remains the major susceptibility locus for SCZD10 in these German pedigrees.

4.2 Fine mapping and MLC1 in schizophrenic families from Germany

Meyer and colleagues (2001) described a rare polymorphism, Leu309Met, in the *MLC1* gene. The methionine-encoding variant was found co-segregating with the syndrome in the large pedigree that supports the 22q13.33 locus. Later, Rubie and colleagues (2003) reported another family found with Leu309Met allele and SCZD10. The finding of the 309Met-encoding allele in a second single German family with periodic catatonia by Rubie and colleagues (2003) is intriguing, considering the absence of this variant in all samples throughout the world tested for it so far, and even in Germany. Although they formally excluded *MLC1* as a putative cause of periodic catatonia in this family, it is very interesting that the family in question, F20, also originates from the same German region where the first family described to carry the mutation, F21, lives. Thus, a founder effect for presence of this variant in the two families must be assumed.

Furthermore, there are considerable uncertainties about the mental health state of the members of F20. In their publication, Rubie and co-workers designated the mother, and two of her brethren (one alive, one deceased) of the index patient as "affected", whereas the father - who is confirmed carrier of 309Met - as "unaffected" (Figure 18; ID 890). However, interviews by telephone conducted with the mother revealed that neither she was psychiatric inpatient, nor were her siblings. Likewise, the father, being carrier of the rare variant, suffers from vascular or mixed dementia with onset at the age of 60 years; he was once hospitalized in a psychiatric department and currently resides at a nursing home. Family members also stated that the father, prior to the onset of dementia, displayed similar symptoms as the son; e.g. aggressiveness and excursiveness. Additionally, considerable nervousness and

restlessness was recorded in the hospital files. In the interview conducted with the mother, she stated that she family members agreed that her son "has got it from the father".

Indeed, there are cases where patients were initially believed to be demented and later on diagnosed with bipolar disorder. The clinical features of dementia are not well described in bipolar patients; however, many patients resemble typical Alzheimer or vascular dementia patients. Patients with profound depression may appear to have dementia, frequently referred to as depressive pseudodementia. Moreover, pseudodementia can also be caused by other mental illnesses such as major depressive disorder and psychosis (Casey and Fitzgerald, 1988; Koenigsberg, 1984; Wright and Silove, 1988; Kessing and Andersen, 2004).

Together, the rationale for considering the father as "unaffected" and the mother as "affected" is not evident from our point of view, and the allocation appears to be at least ambiguous. - Rubie and colleagues did not comment on these conditions in their paper. Thus, careful re-examination of members of F20 by independent clinicians is recommended to reveal whether the psychoses reported from different members of this family are in fact related. These controversies underscore the importance of exact definition of the phenotype in question. Periodic catatonia, which was the marker trait in the study by Rubie and colleagues, constitutes a disease with variable penetrance. However, as described by Leonhard, following akinetic or hyperkinetic crisis typically leading to hospitalization and neuroleptic treatment, the disease usually terminates in a defective state covering blunting of affects as well as increased irritability. Thus, the presence of periodic catatonia seems less likely in the mother, who was caregiver to both her spouse and son for a long time, than in her husband. Furthermore, *MLC1* might not be a susceptibility gene in a narrower sense, but rather represent a modifier gene (Fanous & Kendler, 2005), thus exerting effects on domains

like impulsivity and cognitive impairment, while another gene influencing psychomotor symptoms might be operational in F20.

4.2.1 MLC1 in Megalenchephalic Leukoenchephalopathy with subcortical Cyst

MLC (OMIM #604004) is an autosomal recessive disorder characterized clinically by macrocephaly, deterioration in motor functions, cerebellar ataxia and mental decline (Van der Knaap *et al*., 1995; Singhal *et al*., 2003). Magnetic resonance imaging (MRI), together with clinical findings, is applied to diagnose the disease. MRI showed evidence of severe white matter involvement and cysts in the tips of the temporal lobes and frontoparietal subcortical area. Slow progressive ataxia and spasticity developed, while intellectual functioning was preserved for years after onset of the disorder.

Until recently, 26 distinct mutations have been identified in this gene, including missense mutations, insertions, deletions and alterations in the splice sites (Leegwater et al., 2001, 2002; Tsujino et al., 2003; BenZeev et al., 2002; Patrono et al., 2003). Some mutations are quite frequent in certain populations, indicating a founder effect (Tsujino et al., 2003; BenZeev et al., 2002; Gorospe et al., 2004). Teijido and colleagues (2004) validated the functional effect of missense MLC1 mutations found in patients with MLC, namely T118M, G212R, S280L and C326R. These mutations dramatically reduced expression of MLC1.

However, only about 60% of patients with the characteristic features of MLC harbour mutations in MLC1. The remaining patients have no mutations in *MLC1*, and linkage with the *MLC1* locus is excluded (Blattner *et al*., 2003; Patrono *et al*., 2003). Teijido's group also found that MLC1 is not expressed in oligodendrocytes, which implies that the myelination defects observed in MLC patients are not directly related to oligodendrocyte dysfunction. This finding implies that there is at least a second gene responsible for the disease (Blattner

et al., 2003). Furthermore, there is intra-familial phenotype variability, indicating that unknown environmental or genetic factors influence the severity of the disease (Teijido et al., 2004).

To date, MLC and schizophrenia have been observed neither co-segregating, co-inciding, nor showing a clinical overlap (Rubie et al., 2003). Thus, the different neuropathology of schizophrenia and MLC is suggested.

Meyer and colleagues (2001) described a rare polymorphism, Leu309Met, in the *MLC1* gene. The methionine-encoding variant, which was not present in the controls, was found co-segregating with the syndrome in the large family that supports the 22q13.33 locus. While several other studies reported that the Leu309Met polymorphism did not exist in their samples (Kaganovich et al., 2004; Leegwater et al., 2002; Devaney et al., 2003, McQuillin et al., 2002), Rubie and colleagues (2003) found the rare methionine-encoding variant again in a single German patient with periodic catatonia. In their study, the rare variant was not present in 270 healthy blood donors and additional 140 index cases. The authors stated that some of the patient's relatives also suffered from psychoses, but were not carriers of the methionine-encoding variant. Thus, they formally excluded *MLC1* as a putative cause of periodic catatonia.

However, in a recent paper, Verma and associates (2005) stated that they have found three rare *MLC1* polymorphisms, Val210Ile, Leu308Gln, and Arg328His, in six subjects from a patient sample with manic-depressive disorder from India. Of these, only Val210Ile was also found in a single subject from the healthy controls. One of the polymorphisms in the Indian population, Leu308Gln, is also located in the polyleucine motif present in the putative S6 region of the protein, adjacent - only two base pairs upstream on DNA level - to

the Leu309Met-encoding polymorphism described by our group (Meyer et al., 2001). Selch and colleagues (2007) replicates Verma´s group other finding that the *MLC1* intronic SNPs rs2235349 and rs2076137 are associated with schizophrenic psychoses. However, both SNPs were specifically only associated with periodic catatonia. Considered the close relationship between periodic catatonia and bipolar disorder (Taylor and Fink, Fink and Taylor, 2003), the results from both groups further support the involvement of *MLC1* in pathogenesis of psychosis. These results underscore the notion that MLC1 variation influences the susceptibility toward periodic catatonia.

Severinson and colleague (2006) previously reported association between microsatellite markers located at 22q13.31-qtel for both schizophrenia and bipolar disorder in a Faeroese sample. This group found an association of rs138880 C-allele, an SNP located in the promoter of *BRD1*, for schizophrenia, bipolar disorder and the combined group. No association was found for *MLC1* SNPs (including Leu309Met) with psychoses in this Faeroese sample. We have investigated this rs138880 SNP in our pedigrees that showed linkage to chromosome 22q13.33 (F20 and F21). However, no linkage of rs138880 to SCDZ10 was found, and hence exclusion of the gene, in pedigree F20 and F21 from this study. Furthermore, the same group has also reported that there was no significant LD between *BRD1* and *MLC1*. Thus, strengthens *MLC1* as a candidate gene for psychoses in pedigree F20 and F21.

While some missense mutations in *MLC1* from MLC patients are shown to be dramatically reducing MLC1 protein expression in the plasma membrane, the 309Met variant did not alter the amount of total protein, its assembly in the endoplasmic reticulum, and the localization in the plasma membrane (Teijido et al., 2004). With respect to these

findings, it should be noted that most *MLC1* mutations other than missense in MLC cause protein trunctation and frameshift (Leegwater et al., 2001, 2002; Tsujino et al., 2003; BenZeev et al., 2002), with the exception of Δ(L303-L304) causing in frame deletion (Rubie et al., 2002).

Speculatively for MLC1, it seems like the mutations putatively involved in psychiatric disorders do not alter protein expression and overall structure, while the mutations causing MLC do repress the expression and truncate the protein. The tendency that MLC patients are showing severe symptoms at relatively young age (Pascual-Castroviejo et al., 2005) implies that somehow the mutations involved in MLC affect the protein function more severely compared to the mutations found in schizophrenia. No functional analysis has been done to reveal putative functional consequences of the Leu308Gln mutation found in the family from India. Therefore, it would be interesting to see whether this mutation also alters the protein expression to test the above hypothesis, while this position is conserved throughout evolution.

Comparative analysis on MLC1 from human, dog, chicken, mouse, chimpanzee, crab-eating monkey, frog and zebrafish also revealed that the amino acid in position 309 found in dog, chicken, frog and zebrafish is methionine like in schizophrenic patients, while the mouse and crab-eating monkey have leucine instead just like in healthy humans. Thus, Leu309Met may represent an atavistic back mutation that is not appropriate in humans with respect to function anymore.

MLC1 is likely involved in distal astroglial processes at the blood-brain barrier and glial-limiting membrane. Aquaporin-4 (AQP4), which is expressed throughout the brain and has a role in osmoregulation, is localized in distal astroglial structures similar to MLC1

(Boor *et al*., 2005; Connoly et al., 1998). Boor *et al.* (2005) suggest further studies on possible interactions of MLC1 with AQP4 and dystrophin-associated glycoprotein complex (DAGC) based on the observation that AQP4 is associated with DAGC and some congenital muscular dystrophies caused by mutations in members of the DAGC. These disorders also show white matter abnormalities by magnetic imaging (Teijido *et al.* (2004) suggest that axonal expression of MLC1 in cortical and hippocampal neurons or in astrocytes close to neuronal processes may also support a role in synaptic transmission and in line with the fact that epilepsy has been found in most of the patients affected by MLC or Alexander disease.

Fatemi *et al*. (2005) induced a viral infection to pregnant mice and found that AQP4 was downregulated in the affected neonatal brain by using microarray technology. Some of the accumulated published data show correlation between brain biochemical, structural, and behavioral indices in the virally exposed mouse progeny and those seen in schizophrenic and autistic subjects (Fatemi, 2005; Harrison and Weinberger 2005; Palmen et al., 2004). However, AQP4 is not specifically addressed yet. Quantitative ImmunoGold microscopy revealed that the density of AQP4 along the perivascular membrane domain of astrocytes was reduced by 44% in the human epiteptogenic hippocampus, suggesting the plausible deficient water and K+ homeostasis implicated in the generation of seizures in patients with mesial temporal lobe epilepsy or MTLE (Eid *et al*., 2004). Interestingly, Qin *et al*. (2005) found that both family history of epilepsy and family history of psychosis are significant risk factors for schizophrenia and schizophrenia-like psychosis. Furthermore, the increased risk for schizophrenia or schizophrenia-like psychosis does not differ by type of epilepsy, and people with a history of epilepsy are at increased risk for schizophrenia and schizophrenia-like psychosis. Thus, if MLC1 function is proven to be related to AQP4, hypotheses leading to MLC or schizophrenia are both plausible.

A collaborative study of bipolar disorder (Ferreira MAR and 62 others, 2008) that combines data from two previously published genomewide association studies (GWASs) with a new GWAS associates two genes that encode components of voltage-gated ion channels with the illness. These findings raise the possibility that bipolar disorder may partly result from channelopathies, disruptions in ion channel subunits or other channel-related proteins. Such molecular lesions underlie a diverse group of disorders, including central nervous system disorders such as epilepsy, migraine, and ataxia. With the bright diagnostic line between schizophrenia and bipolar disorder, first drawn by Emil Kraepelin, is increasingly called into question as has been pointed out by Nick Craddock and Michael Owen, the emergence of overlapping candidate genes and the similarity between the psychotic phenotype seen in the manic phase of bipolar disorder and in schizophrenia suggest that there will be a growing cross-fertilization in genetic research in these two arenas (Craddock and Owen, 2005). This GWAS result suggested that channelopathies might play a role in schizophrenia as well. This is an issue that has not previously received much attention but obviously now warrants specific investigation. Therefore, it is important to emphasize once again that our periodic catatonia candidate gene, *MLC1*, is possibly a non-selective cation channel which exhibits weakly structural similarity to the voltage-gated potassium channel Kv1.1 (Meyer *et al*., 2001, Leegwater *et al*., 2001).

Chapter 5
Concluding Remarks and Outlook

5.1 Outlook

5.1.1. Whole-genome association study (WGAS or GWAS) for mental disorders

Whole-genome association analysis is one of the latest tools geneticists employ to identify susceptibility genes for complex disorders. The technique uses extremely dense DNA arrays, or chips, to simultaneously detect hundreds of thousands of single nucleotide polymorphisms (SNPs) in DNA extracted from blood samples.

Previous studies have focused on the technique of genetic linkage, but new technologies and experimental resources make whole-genome association studies more feasible. Association studies of this type have good prospects for dissecting the genetics of common disease, but they currently face a number of challenges, including problems with multiple testing and study design, definition of intermediate phenotypes and interaction between polymorphisms. Large-scale studies like GWAS provide optimal power to detect genetic variants that influence susceptibility broadly across the mental disorder phenotype and across populations. It is, however, true that they may not be the optimal approach for detecting susceptibility variants that are either specific to certain populations or confer risk specifically to certain aspects of the clinical phenotype. In such situations, specific signals could be washed out. It should also be noted that the current GWA technologies, despite covering 500,000 SNPs, are still sampling just a small fraction of the genome, and were not designed for specific coverage of these gene candidates, which consequently are not well

covered. Some suggested that it would take in the range of two million SNP chips to densely cover the top current candidates.

A collaborative study of bipolar disorder (Ferreira MAR and colleagues, 2008) that combines data from two previously published GWASs with a new GWAS associates two genes that encode components of voltage-gated ion channels with bipolar disorder. The strongest association ($P = 9.1 \times 10^{-9}$) was found in ANK3 (Ankyrin-G) on chromosome 10q21, a gene that is required for the clustering of voltage-gated sodium channels at axon initial segments (Zhou et al., 1998) and nodes of Ranvier (Poliak and Peles, 2003), a configuration that underlies the rapid and efficient propagation of action potentials along myelinated axons. The second-strongest association was at rs1006737, in the third intron of CACNA1C ($P = 7.0 \times 10^{-8}$). A third association was found near C15orf53, a gene on 15q14 of unknown significance. No differential associations for these three regions were found across bipolar disorder subtypes, presence of psychosis, age of onset, sex, or response to treatment.

These findings raise the possibility that bipolar disorder may partly result from channelopathies, disruptions in ion channel subunits or other channel-related proteins. Such molecular lesions underlie a diverse group of disorders, including central nervous system disorders such as epilepsy, migraine, and ataxia.

Preliminary findings from the GWASs and schizophrenia studies showed that a lot of signals or candidate genes were shared for both disorders, as has been shown through other method (Figure 21). Following these recent progress mainly in the fields of genetics and neurobiology, the validity of the diagnostic distinction between schizophrenia and bipolar disorder is increasingly challenged.

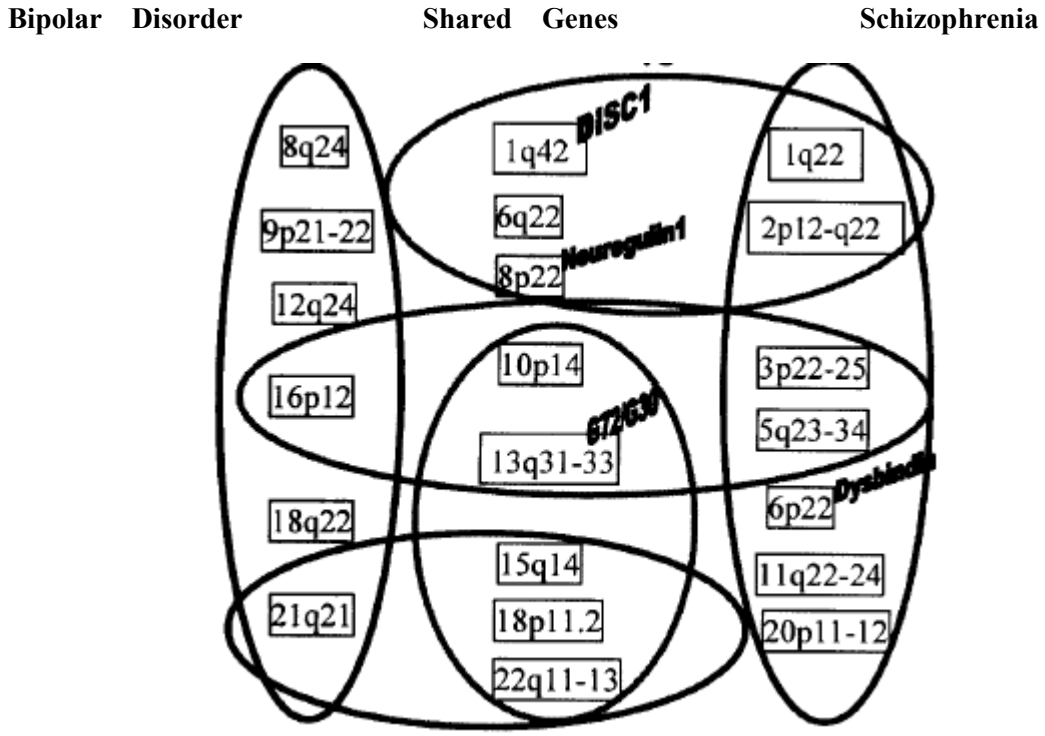


Figure 21. An illustration of the shared genes between bipolar disorder and schizophrenia. Note that 15q14 locus is a shared locus for both illness (graph taken from http://www.psycheducation.org, 2008).

5.1.2. Whole-Genome Homozygosity Association (WGHA)

A new type of genetic analysis has uncovered novel risk factors for schizophrenia. Lencz and colleagues (2007) reported that specific runs of homozygosity (ROH), regions of DNA where individuals inherited the identical material from both parents, are more prevalent in patients with schizophrenia. Some of these runs contain or lie close to genes that have been previously implicated in the disease, while others may harbor new genetic risk factors. The new analysis, called whole-genome homozygosity association (WGHA), is better able to find associations with recessive alleles than traditional methods.

Whole-genome homozygosity association is an extension of the whole-genome association. But the group has taken WGA technology one step further. Instead of focusing on single SNPs, it has been used to look for clusters of SNPs that show no variation in a

given individual, then ask if any of these homozygosity runs are related to disease, in this case schizophrenia.

Being homozygous means that the same allele has been inherited from both parents, which is the case in many simple autosomal recessive disorders. In general, such disorders are much rarer than schizophrenia, occurring in about 0.01 percent of the population (for schizophrenia, estimates around 1.0 percent), and studies on genetic transmission suggest that schizophrenia does not follow a simple Mendelian pattern. It is more likely that ROHs may be overrepresented in schizophrenia and that the disease might particularly relate to those regions of the genome and thus lead us to do further investigation in the region of interest.

5.1.3. Non coding RNA role in psychiatric disease

A series of large scale transcriptome studies leave no doubt that the mammalian transcriptome is characterized by a complex mosaic of overlapping, bi-directional transcripts and a plethora of non-protein coding transcripts arising from the same locus. This newly discovered complexity is not unique to mammals. Similar high-throughput studies in invertebrate animals (He et al., 2007) demonstrate the generality of the mammalian genome organization among higher eukaryotes. Even in bacteria, an unexpected complexity of regulatory RNAs was discovered in recent years (Gottesman, 2004).

5.1.3.1 Small nucleolar RNAs (snoRNAs)

Small nucleolar RNAs (snoRNAs) represent one of the most abundant classes of ncRNAs. They act as guides for single nucleotidemodification in nascent ribosomal RNAs and other RNAs in the nucleolus of eukaryotic species. While no targets are present or

known for so-called *orphan snoRNAs*, most guide snoRNAs target ribosomal RNAs (rRNAs) or small nuclear RNAs (snRNAs). Recently, there has been a report that several snoRNAs may also target tRNAs and other snoRNAs (Zemann et al., 2006). The orphan snoRNAs have been implicated in modulating alternative splicing (Bazeley et al., 2008). One evidence of snoRNA influence in brain functioning came from the Prader-Willi syndrome (PWS). The Prader-Willi syndrome is a congenital disease that is caused by the loss of paternal gene expression from a maternally imprinted region on chromosome 15. This region contains a snoRNA, HBII-52, that exhibits sequence complementarity to the alternatively spliced exon Vb of the serotonin receptor 5-HT_{2C}R. Loss of expression of the human homologue (HBII-52) in Prader-Willi syndrome causes alterations in *5HTR2C* pre-RNA processing, which may underlie some of the neuropsychiatric problems in these individuals (Kishore and Stamm, 2006).

5.1.3.2 mRNA-like ncRNAs (mlncRNAs)

A rapidly growing class of ncRNAs looks like protein-codingmessenger RNAs in many respects. These mRNA-like ncRNAs (mlncRNAs) are transcribed by polymerase-II, polyadenylated at their 3' end, capped with 7-methylguanosine at the 5' end, and typically spliced. While huge numbers of mlncRNAs were found in both animals and plants, next to nothing is known about most of them. There is, however, mounting evidence that many of them are associated with diseases such as :

SZ-1/PSZA11q14↓ in schizophrenia (Polesskaya et al, 2003); *DISC2* in schizophrenia and bipolar affective disorder (Millar et al, 2004; Chubb et al, 2008) and *SCA8* in Spinocerebellar ataxia type 8 (Mutsuddi et al., 2004).

5.1.3.3 MicroRNAs (miRNAs)

MicroRNAs (miRNAs) are a recently discovered class of small RNA molecules implicated in a wide range of diverse gene regulatory mechanisms. Interestingly, numerous miRNAs are expressed in a spatially and temporally controlled manner in the nervous system. This suggests that gene regulation networks based on miRNA activities may be particularly relevant in neurons. Recent studies show the involvement of RNA-mediated gene silencing in neurogenesis, neural differentiation, synaptic plasticity, and neurologic and psychiatric diseases.

Microdeletions at chromosome 22q11 that are associated with schizophrenia in humans alter the production of gene-regulating microRNAs in mice (Stark et al., 2008) and responsible for changes in miRNA expression and contributing to the behavioral and neuronal phenotypes in mice. The work is the first time that microRNAs have been implicated in cognitive dysfunction associated with schizophrenia, and may provide a new handhold on the complex molecular basis of the disease.

The significance of this work is that it implicates a completely novel, previously unsuspected group of susceptibility genes and brings investigators a step closer to understanding the biological mechanisms of this disorder. Implication of such a large family of genes (the most recent estimate puts the number of human microRNAs at at least 400 that influence the expression of as many as a third of all genes) could partly account for the genetic complexity associated with this devastating disorder and explain some of the difficulties that has been encountered in the efforts to pinpoint individual genes.

5.1.4. Elucidation of MLC1 function

The mouse has become a critical model in studying human disease because scientists have access to many inbred strains, each expressing distinctive physiological and behavioral characteristics. Researchers can now insert, knock out, or mutate mouse genes, quickly breed a generation that expresses the change, and then see how it affects behavior. When illness-linked genes are discovered, they will be inserted and expressed in mice to find out what they do at the molecular, cellular and behavioral levels. Thus, we will be able to track a wiring abnormality, a cell migration abnormalities.

The study from this dissertation has generated a knockout vector for *Mlc1* for making the totally null knockout mouse. On the other hand, it will be important and very interesting to generate another Mlc1 knockout vector that will be tissue-specifically inactivated. Both models then should be characterize from a morphometric,biochemical and neurodevelopmental point of view. This will allow us to understand the role of MLC1 in different tissues and the bases of the clinical symptoms of the disease, as well as to test in vivo possible therapies.

In the study for this dissertation, a first attempt in elucidating MLC1 function was approached by using yeast two-hybrid system for finding the protein binding partner(s). Given the nature of MLC1 as an integral membrane protein, finding its binding partner(s) through the split-ubiquitin system (Johnsson and Varshavsky, 1994) and its adaptations for protein-protein interaction in yeast (e.g : Dualmembrane system from Dualsystem AG) would be a better approach in the future. Proteins with several transmembrane domains has been shown good results using this system, take ABC transporter of *S.cerevisiae* as a striking example with 17 transmembrane domains (Paumi et al., 2007).

5.1.5 Functional glycomics

Carbohydrate structures are very much a part of the language of life. Some even call carbohydrates the third alphabet, behind DNA and proteins. Glycosylation, the attachment of carbohydrate chains to proteins, is a crucial part of biology, and it is estimated that half of all proteins encoded by the human genome get sugars attached to them at some point after they are made. All cells are covered with carbohydrates.

Though they are not charged with storing genetic information like DNA or acting as enzymatic workhorses like proteins, carbohydrates nevertheless do carry information and are responsible for important biological functions, playing a central role in many types of intercellular communication events, protein folding, cell adhesion, and immune recognition.

One of the most important frontiers of basic research in biology today is to understand the human glycome-all of the types of carbohydrate structures in the human body and what they do. This is a profoundly difficult efforts. The total number of carbohydrate structures in humans may range from 10.000 to 20.000.

Several laboratories in the NIGMS-funded consortium have collaborated to create a new glycan array, which will make it easier and faster to determine how a diversity of human glycan binding proteins interact with carbohydrates in biological systems. Over the last few years, consortium scientists have constructed a library of more than 200 biologically relevant sugars. They have developed a simple way to permanently array sugars onto glass slides. Using standard commercially available technology, sugars that have been given an "amino linker" are printed onto slides coated with a chemically reactive surface and become covalently attached. The array of more than 200 different sugar structures can be expanded as additional sugars become available.

For the majority of glycan structures, their "inner core" portion, is the same in all cells and tissues. What varies are the terminal sequences of sugars, the last few end sugars on the carbohydrate chain. Proteins that bind to carbohydrates only recognize the last few sugars in a carbohydrate chain, and perhaps as few as 500 or so of these terminal sequences in nature are relevant.

In the past years, Blixt and colleagues (2004) looked at the carbohydrate binding properties of antibodies using the array, suggesting this new tool may be developed into a powerful diagnostics screen for evidence of antibodies in human blood that would indicate a bacterial or viral infection, a hidden cancer, or an emerging autoimmune disease. Giving the complexity of psychiatric diseases, it will not be surprisingly that in the near future this field will become an important new research avenue.

5.2 Concluding remarks

Nature determines the complexity of disease etiology and the likelihood of revealing disease genes. While culprit genes for many monogenic diseases have been successfully unraveled, efforts to map major complex disease genes have not been as productive as hoped. The conceptual framework currently adopted to deal with the heterogeneous nature of complex diseases focuses on using homogeneous internal features of the disease phenotype for mapping. However, phenotypic homogeneity does not equal genotypic homogeneity.

Rapid developments of the genotyping and gene-expression technologies are now enabling cheaper, faster and more precise massive high-throughput research. There has been great anticipation in the world of psychiatric research over the past year, with the community awaiting the results of a number of genome-wide association studies (GWASs). However, the

results to date still raised the question of why there was no replication of popular candidates such as *NRG1* or *DTNPB1* or *DISC1*, altough there was a preliminary support for *NRG1* and *DISC1*. In this regard, linkage studies will remain the valuable preliminary effort for systematic molecular genetic research in psychiatric disease for sometime in the forseeable future.

Encouragingly, the 15q14-15 and 22q13.33 chromosomal regions have repeatedly been implicated in autism, bipolar and schizophrenia by independent groups from different populations, suggesting that true linkage signals may have been uncovered. Moreover, the latest result from bipolar GWASs associates genes that encode components of voltage-gated ion channels with the illness, with one of the highest signal being in 15q14 near *C15orf53*, the hypothetical gene residing in the fine-mapped region B for periodic catatonia from this dissertation. These latest GWAS findings (Nature Genetics, advance online publication from 18 August 2008) thus support findings from this dissertation and raise the urgency to analyse further this particular gene with unknown significance in our German pedigrees. The other important point is that this dissertation and the afforementioned GWAS results give more support to the recently growing discussion in the field of psychiatry, that is the argument that schizophrenia and bipolar disorder share too many symptoms and genes to justify defining them as distinct syndromes.

The identification of the actual disease-predisposing variant in the usually large regions is a challenge that requires a combination of genetic and biological strategies and hypothesis. For example, the involvement of epigegenetic factors such as DNA methylation, histone deacetylation, chromatin modification, RNA interference, RNA editing and DNA rearrangement have long been proposed in complex disease. Since epigenetic processes in-

crease the complexity of genomic responses by allowing shortterm fine-tuning of the genome, and provide a mechanism for gene x environment interaction, it is set to emerge as an important avenue of research.

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Appendix

Eidesstattliche Erklärung Trier, im November 2008

Hiermit versichere ich, dass ich die vorliegende Arbeit selbstständig verfasst habe und keine anderen als die angegebenen Hilfsmittel benutzt wurden.

Savira Ekawardhani