# Characterization of DMWD and its role in Myotonic Dystrophy (DM1)

# Characterization of DMWD and its role in Myotonic Dystrophy (DM1)

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van de Medische Wetenschappen

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For Nita

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# Chapter 1

## General Introduction

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*1.1 Myotonic Dystrophy, the clinical picture*

Myotonic Dystrophy (DM) (Steinert disease, also referred to as Myotonia atrophica, Dystrophia Myotonica, Myotonic muscular dystrophy) is a dominantly inherited multisystemic disorder and the most common of adult muscular dystrophies. DM was first described in 1909 by Steinert and Batten & Gibb [14,206]. The disorder has a very complex clinical picture with a wide range of symptoms and a variable age of onset, which can be anywhere between birth and over 60 years of age [83].

Myotonic dystrophy patients may be asymptomatic, have minimal features (e.g. cataract and asymptomatic myotonia), show moderately severe facial and distal limb muscle wasting and weakness, or have a severe congenital disorder with hypotonia, respiratory insufficiency and mental retardation [83]. In general, the earlier the manifestation of the disease occurs in life, the more severe the symptoms are. DM affects many different tissues and organs such as distal limb muscles and facial muscles, smooth muscles (uterus and intestine), the heart, the eye, the brain and testis [83,148,179,232]. Another feature that is often seen in Myotonic Dystrophy is anticipation; the progressively earlier appearance in successive generations accompanied by more severe symptoms. Based on the time of onset and the phenotypic appearances, Myotonic Dystrophy patients can be divided in three groups: the first group is the group of patients with late onset and mild symptoms, which usually only comprises cataract; the second group is the category of patients with adult onset, and more severe symptoms like myotonia and muscle weakness, cardiac conduction defects, smooth muscle impairment in the gastrointestinal tract, mental changes, testicular atrophy and endocrine malfunction; the third group consists of patients with congenital or very early onset, with hypotonia and facial displegia, jaw weakness, respiratory and feeding difficulties and mental retardation [71,92,148,227,232]. In 1992 the mutation underlying Myotonic Dystrophy (an expanded $(CTG)_n$-repeat) was located and identified [7,24,26,56,81,101,137]. Shortly after the discovery of this mutation a category of DM patients with similar disease manifestation as DM1 patients (called PROMM or DM-type ) but without an expanded $(CTG)_n$-repeat were identified. Muscle problems in patients with this form of Myotonic Dystrophy occur usually at more proximal sites, and there is no type 1 (slow) fibre predominance (instead now mainly type II fibres are affected). Congenital manifestation and clear anticipation is also not seen. Moreover, DM2 patients do lack the psychological/social and hypersomnia problems ("sad appearance") of DM1 patients [148,176,208]. Moreover, the severe central nervous system involvement and the broad correlation between age of onset of the disease and the repeat size, as can be seen in DM1 patients, were not reported for DM2 patients [148,175]. In 1998 a second Myotonic Dystrophy locus was reported for this class of patients [177].

*1.2 Mutation underlying Myotonic Dystrophy type 1 (DM1)*

The primary defect underlying Myotonic Dystrophy type 1 is a mutation in the polymorphic $CTG_{5\text{-}37}$ trinucleotide repeat located in the middle of a gene dense area at chromosome region 19q13.3, which caused the repeat to increase [7,24,26,56,81,101]. This repeat is located in the 3'-UTR of the Myotonic Dystrophy Protein Kinase gene (DMPK) [24,56,101]. The size of the CTG-repeat measured in DM-patients is broadly correlated with the severity and age of onset of the disease (reviewed by [232]). In the regular population the repeat varies in length between 5 and 37 CTGs but if the repeat length is above 35 it is prone to instability [25]. DM patients with mild symptoms of the disease usually have CTG-repeats in between 37 and 180 triplet-units, while DM patients with adult onset of the disease have a CTG-repeat of 200 to 1000 units and the congenital DM patients can have CTG repeats as large as 5000 units or longer. In more than 80% of parent-child pairs, the age of disease onset was earlier in life and the repeat size was greater in the offspring than in the parents [71].

Variation in repeat size is not only found within generations (intergenerational), but also in somatic tissues. When repeat expansions in blood DNA of DM patients were analyzed, it was shown that there was somatic heterogeneity of the expanded allele [23,24,137,232]. Even in cultured cells derived from several different tissues of DM patients, the mitotic instability of the CTG repeat was detected. Different cell lineages resulted in distinct length-mosaicism in different cell lineages [102,124]. When CTG-repeats in sperm and blood of male DM patients (with mild symptoms) were analyzed, mosaicism was found in both blood and sperm, but with greater variation in sperm. However, severely affected males with large expanded alleles in blood had similar or smaller repeats in sperm or lacked expanded signals completely and alleles greater than thousand CTGs were not found in sperm [102]. This suggests that there may be a barrier to transmission of long CTG-repeats at some point in spermatogenesis.
Jansen and co-workers also reported several cases where the length of the repeat in blood of the offspring was much larger than the length of the repeat in the father's sperm [102], suggesting that the repeat expansion takes place during development or growth of the offspring.

*1.3 Mutation underlying Myotonic Dystrophy type 2 (DM2)*

The mutation responsible for DM2 at chromosome 3q21 was found to be a CCTG repeat expansion located in the intron of the gene for zinc finger protein 9 (ZNF9) [131]. The repeat is transcribed but excised from the primary transcript by splicing and therefore not translated, similar to the repeat in the DM1 locus. The ZNF9 protein is thought to be a RNA-binding protein [168,174], which is most abundantly expressed in heart and skeletal muscle [131]. The ZNF9 gene (DM2 region) appears to be unrelated to any of the genes encoded in the DM1 region. Similarly, genes in the DM2 region – KIAA1160, Rab 11B, glycoprotein IX, FLJ11631 and FLJ12057- appear to be unrelated to any of the genes in the DM1 locus [175]. The range of expanded repeat length found in DM2 patients is extremely broad, from 75 to more than 11,000 CCTG repeats with a high degree of somatic mosaicism [131]. It is of note here that no clear correlation has been found between the length of the CCTG repeat and severity of disease manifestation (symptoms in a family with 75 repeat elements is highly similar to those in families with thousands of units in length [Day and Ranum, unpublished]).
It remains unclear how the $(CTG)_n$- or $(CCTG)_n$-repeat expansions can cause very similar, but clinically distinct DM1 and DM2 features, although various hypotheses have now been forwarded (see below).

*1.4 Triplet repeat diseases*

DM1 is not the only disease that is caused by a triplet repeat. To date, 16 trinucleotide-expansion diseases have been reported [141], including the FRAXA and FRAXE syndromes, Kennedy's and Huntington's disease, and different forms of spinocerebellar ataxia (SCA 1-3, 6-8) [179]. Next to these trinucleotide expansion diseases there is the Myotonic Dystrophy 2 tetranucleotide [131] and the SCA10 pentanucleotide disorder [144].
The repeats in the triplet repeat diseases are either located in coding regions, specifying stretches of the same amino acid in the normal protein (often polyglutamine repeats as in Huntington's disease, Kennedy disease, SCA 1-3, 6 and 7), or in non-coding areas (FRAXA, FRAXE, SCA8 and Myotonic Dystrophy type 1) [142,179].
A common feature of the proteins with amino acid stretches is that they are involved in neuronal dysfunction or neurodegeneration. It has been well documented that expanded polyglutamine fragments, cleaved from their respective full-length proteins, form microscopically visible oligomeric aggregates in affected individuals. This, in turn leads to cytotoxic effects, with loss of normal cellular protein synthesis and degradation functions and ATP depletion. How the polyglutamine oligomers exert this role is still unknown [142].

The other group of triplet repeat related diseases, with triplet repeat expansion in non-coding regions, do not seem to share one common pathobiological mechanism. Rather, here the nature of the triplet and the location of the repeat within the gene seem to dictate disease aetiology [142]. For instance, SCA8 is a dominantly inherited, slowly progressive spinocerebellar ataxia characterized by reduced penetrance, which is caused by a transcribed but untranslated $(CTG)_n$-repeat located in the 3'-UTR of the SCA8 gene [39,118,207,236]. SCA8 transcripts are almost exclusively expressed in the brain, which is consistent with the central nervous system involvement of this disease [160]. On the other hand, in DM1 the $(CTG)_n$ bearing DMPK gene transcripts are much more widely expressed, which is consistent with the multisystemic features and the very complex clinical picture of DM.

*1.5 Hypotheses*

Three distinct hypotheses have been postulated to explain how a triplet repeat in the DMPK UTR region can cause such a complex clinical picture.

The first hypothesis is based on an altered processing of the DMPK RNA due to the expanded repeat. Several reports have shown that presence of the expanded (CTG) repeat in the DMPK gene results in diminished expression of DMPK mRNA and retention of the transcripts in the nucleus, which prevents proper translation in the cytoplasm [38,170,216,227]. The mutational expansion is thus thought to cause haplo-insufficiency and result in a partial deficiency of the DMPK protein. Two groups have used knockout mice models to examine if DMPK gene loss would mimic these effects. Their analyzes showed that older homozygous DMPK knockout mice did develop a mild myopathy, but DMPK heterozygous mice did not show any muscle pathology [100,180]. Therefore it was concluded that the lack of DMPK protein was not the principal cause of the myopathy seen in patients.

The second hypothesis argues that the CUG repeat in the DMPK RNA has a toxic gain-of-function effect on cellular metabolism. Mechanistically, it is thought that CUG repeats precipitate the formation of nuclear ribonuclear protein inclusions, which in turn may play a trans-acting role in obstructing RNA processing or transport. This hypothesis was strengthened by a report of Mankodi and co-workers who showed that mice expressing an expanded CUG repeat in the 3'-UTR of the human skeletal actin gene (HSA) produced the myopathy and myotonia characteristic of DM patients [139]. This finding suggests that CUG repeats have a toxic gain-of-function effect on cell metabolism regardless of the gene where the CUG is located in. Other evidence includes the fact that the expression of mutant DMPK mRNA containing an expanded repeat in the 3'-UTR inhibits differentiation of cultured myoblasts [6]. Tapscott proposed in his report [211] that there would be a general mechanism of RNA pathogenesis in which CUG-repeat-containing DMPK transcripts that accumulate as nuclear RNA foci alter the regulation of CUG-binding proteins, like CUB-BP [214] and muscleblind [151]. There is evidence that alterations in CUB-BP, induced by the CUG-repeat, results in the altered RNA splicing of several genes, including cardiac troponin T [170], the insulin receptor (IR) [189], the chloride channel CIC-1 [30] and the tau protein [193]. Alterations in the splicing of the IR lead to a predominance of the insulin-resistant splice form, which is seen in both DM1 and DM2 [131,189].

Alterations in CIC-1 mRNA processing are also shared between DM1 and DM2, suggesting a common RNA-based mechanism for disease manifestation. Still, various questions remain. It is currently unclear how the "magnitude" of formation of ribonuclear protein inclusions in DM1 and DM2 relates to the RNA processing problems, for example CUG-BP itself cannot be found in inclusions and clear evidence for a correlation between the magnitude of inclusion formation and the disease manifestation is also not available.

Although there is now broad consensus over the toxic RNA-gain-of-function hypothesis, it has been widely accepted that the down-regulation of DMPK and the possible effects of repeat expansion on neighbouring genes also may be involved in aspects of disease manifestation. To this picture fits a third hypothesis, which postulates that the expanded CTG repeat in the DMPK gene might alter expression of not only the DMPK gene but also neighbouring genes like SIX5 and DMWD. Otten and co-workers provided support for this model by showing that expanded CTG repeats in DM-patients changed the chromatin structure in the DMPK region. This could affect more genes than just the DMPK gene [165]. One of the genes that could be affected is Six5 as Klesert and co-workers reported that expression of Six5 RNA in cells derived from patients was decreased compared to non-DM-affected controls [114]. In addition two groups reported that mice that are deficient in Six5 develop cataracts [113,187]. Finally, for this study it is important to note that in DM1 patients with large CTG-repeats expression of the DMWD gene from the DM1 locus was lowered, with repeat-expansion size being correlated inversely with DMWD expression levels [5,48], also see [55,77].

*1.6 The DMPK area*

The DMPK gene on 19q is situated in a gene dense area and is directly flanked by two genes: the sine oculis homeobox gene 5 (Six5; earlier called DMAHP) [21,85] downstream, and the Myotonic Dystrophy WD-repeat gene (DMWD; earlier called DMR-N9 in mouse and gene 59 in human) [98,195] upstream (see Fig. 1).

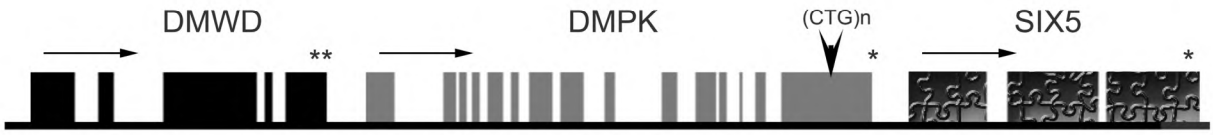


**Fig. 1.** The DMPK region.
The DMPK region with the three genes in this area: DMWD, DMPK and SIX5. Each block indicates an exon and the arrows show transcription start and direction. Polyadenylation signals are marked with * and the location of the repeat is indicated with $(CTG)_n$

*1.7 The DMPK gene*

The DMPK gene covers an area of 14 kb of genomic DNA and is comprised of 15 exons, which includes several regions that can be alternatively spliced and it encodes a protein of 629 amino acids [24,56,71,101,137,195]. The protein consists of several motifs like a leucine-rich stretch at the N-terminal part, which may play a role as aggregation or routing signal in the cell [71]. The protein shares homology with members of the family of cyclic adenosine monophosphate (AMP) dependent protein kinases (N-terminal) and was characterized as a serine/threonine protein kinase with a catalytic domain of approximately 43 kDa [23,101,138,232]. The kinase domain in the DMPK protein is most homologous to the p21-activated kinases myotonic dystrophy kinase-related Cdc42 binding kinase (MRCK) [126] and ROCK/Rho-kinase/ROK [126,143,157], which might suggest that DMPK has a role in actin cytoskeleton remodelling, but direct evidence to support this has not been reported.

The catalytic domain is followed by α-helical domain and a hydrophobic C terminus, which may function as a membrane anchor [24,56,71,72,101,137,195]. The DMPK mRNA is alternatively spliced which gives rise to 6 major isoforms: four of approximately 74 kDa (heart, skeletal muscle- or brain-specific) and two truncated forms of approximately 68 kDa (smooth muscle-specific) lacking the C-terminus. Through the alternative splicing the DMPK gene specifies distinct protein products [72] with different biochemical properties and subcellular distribution (Wansink et al, *in press*).

DMPK expression is found in a wide range of tissues, with the highest expression in organs containing smooth muscle cell linings (stomach and colon) and in cardiac and skeletal muscles. Moderate levels of DMPK expression are also found in brain.

*1.8 SIX5*

SIX5 (also known as DMAHP) is located just downstream at 1.2 kbp of the last exon of the DMPK gene and is a vertebrate homologue of *sine oculis*, a gene essential for the development of the visual system in *Drosophila melanogaster* [32]. Six5 encodes a homeodomain transcription factor and is a member of the *Six* (sine oculis homeobox) family [110]. An enhancer element, that controls the expression of SIX5, was identified within a DNAse I hypersensitive site immediately adjacent to the $(CTG)_n$-repeat. DM alleles with large expansions were shown to lose DNAse I hypersensitivity [114,165], indicating that CTG expansion promotes local chromatin remodelling. A 2-4 fold reduction in the steady-state transcripts levels of SIX5 from the expanded allele, compared to the normal allele of DM1 patients, was seen in fibroblasts and skeletal muscle cells, suggesting that expansion of the $(CTG)_n$-repeat indeed has spreading effects and affects neighbouring gene expression [114].
The murine Six5 RNA is expressed in a wide range of adult tissues including skeletal muscle, heart, testes, brain, smooth muscle, thymus, kidney and liver, as well as in head, limbs, liver, kidney and heart of 12.5 dpc embryos. Transgenic mice containing a promoter construct fused to lacZ showed that the protein is expressed in several neuronal tissues, sensory tissues, some muscles and other tissues like gonadal ridge, kidney tubules and liver [85]. In the human eye SIX5 expression has been reported at low levels in the adult lens, cornea, uvea, retina and sclera but not in the optic nerve or in the fetal eye [235]. Two groups produced knock out mouse models for Six5 and showed that Six5 deficiency or reduced Six5 dosage increases the incidence of ocular cataracts, a feature also seen in human DM [113,187]. Furthermore Klesert and co-workers reported that homozygous SIX5 deficient mice show no apparent abnormalities of skeletal function [113].
These results suggest that epigenetic effects of CTG expansion, which may include chromatin condensation and hypermethylation, alter gene transcription in the vicinity of the repeat tract (DMWD and/or Six5). Research into the boundaries to which such epigenetic effects will spread as a function of the repeat length, may provide insight into the multisystemic nature of the DM phenotype [165,205].

*1.9 DMWD*

The upstream neighbouring gene of DMPK, DMWD is the subject of this thesis study. This gene consists of 5 exons within a region of 7 kb and encodes for a protein of 650 amino acids. The DMWD gene has two weak polyadenylation sites and the most downstream DMWD polyadenylation site is located at 1240 basepairs upstream of the DMPK translation start codon [98]. The RNA and protein products of the DMWD gene are ubiquitously expressed in all adult tissues, but occur most abundantly in testes and brain. Recently, it was shown that DMWD was most profoundly expressed in neuropil of brain areas with a high density of synaptic connections [231].
The onset of expression is very early in mouse embryogenesis starting at 9.5 days post conception [98]. The protein has two interesting characteristics, first it has a proline-rich stretch at its N-terminal end and secondly it contains four so-called WD-repeat units. A relationship between DMWD gene products and the DM phenotype was suggested when two reports came out that showed decreased DMWD expression in DM-patients.

Eriksson et al reported that DM-patients had an inverse correlation between DMWD mRNA expression levels in the cytoplasm and CTG-repeat size; the larger the repeat the lower the DMWD expression [48]. Around the same time Alwazzan et al reported that the DMWD DM-associated allele in the cytoplasm of fibroblast cell-lines derived from DM patients was reduced in comparison with the wild type DMWD allele. This was however not seen in the RNA fractions from the nucleus of those same cells tested, a correlation between repeat size and level of DMWD reduction was also not detected [5].
Groenen and Wieringa reported that under artificial circumstances in a mouse model, when the area downstream of DMWD was changed by a targeted mutation replacing exon 1-8 of the DMPK gene, the DMWD and DMPK transcripts became "contiguous" [71]. This could mean that when the topology of the DMWD-DMPK region is distorted, normal regulation from the DMPK promoter is lost, but that the DMPK transcriptional site can then be opened up by ongoing RNA polymerases exiting from the DMWD transcription unit. This opens interesting novel possibilities for involvement of DMWD in the DM manifestation.
The DMWD protein is highly conserved between species from human to plant (*Arabidopsis thaliana*) and orthologues have been found by computer comparison in *C. elegans* (*C08B6.7*), *S. pombe* (*YDE3*), *A. thaliana* (*T2N18.8*), *D. melanogaster* (*CG6420*), and *A. nidulans* (*CreC*). This last homologue in particular is interesting as recently it was discovered that the CreC protein interacts with another protein, designated CreB, which has a role in metabolic regulation in *A. nidulans* [134]. CreB is a deubiquinating enzyme and has a human homologue called ubiquitin hydrolysing enzyme I (UBHI). CreC most likely stabilizes the CreB enzyme by masking several internal PEST sequences [134]. PEST sequences are hydrophilic stretches of polypeptide sequences that are rich in proline, glutamic acid, serine and threonine, flanked by positively charged residues and are implicated as signals for proteolysis [178,183]. CreB in its turn stabilizes yet another protein, CreA, by removing covalently bound ubiquitin moieties. CreA is a domain DNA-binding regulatory protein involved in carbon catabolite repression [134]. When these findings are extrapolated to the function of DMWD in mouse and human, they bring interesting possibilities for the role of DMWD.

### *1.10 WD-repeats*

One of main featuring characteristics of DMWD is the WD-repeat domain. Most proteins consist of one or more domains, defined polypeptide segments that are conserved during evolution often with a specific catalytic or structural function. G β subunits of heteromeric GTP binding proteins (G-proteins) are the archetypal forms of WD-repeat proteins. They contain seven WD-repeat units and form a dimer with the G γ subunit of large tripartite G-proteins, an association necessary to transduce signals from extracelullar ligand binding across the plasma membrane [62,199,226]. The G β/γ dimer was the first WD-repeat protein whose crystal structure was determined [204]. It was shown that its seven WD-repeats are arranged in a ring to form a β propeller structure with seven blades. The blades themselves consist of a four-stranded twisted β sheet and binding of the γ subunit to the β subunit is essential for correct folding. [123,204,224]. Each WD-repeat sequence is composed of four strands (a-d). A WD-repeat sequence is not linearly equivalent to one propeller blade because each blade is constructed of the first three strands of one repeat (strand a – c) and the last strand of the next repeat (strand d). This arrangement is needed to keep the ring closed and four or more WD-repeats are needed to make a full-circle [199]. Because all WD-repeat proteins have this structural appearance they are placed in one large structural family: the WD-repeat family. Members of the WD-repeat family are identified in many eukaryotes but also in prokaryotes like *Synechocystis sp.*, *Thermomonospora curvata* and *Anabaena sp.*

No identifications of WD-repeat proteins were made in the bacterial genomes of *E.coli, H.influenzae* or *B.subtilis* [60].
WD-repeats are thought to interact with one or more partner proteins. Obvious protein binding sites are the top and the bottom of the β propeller ring, but also the outside of the propeller can interact with proteins. An example of a protein binding with more than one partner is the G β subunit, which interacts not only with the G γ subunit but simultaneously with at least one of approximately 15 other proteins [199]. In humans at least four inheritable diseases are thought to be the result of mutant WD-repeat proteins: lissencephaly caused by a mutation in the lissencephaly-1 gene (LIS1) [27,132,159], the triple-A syndrome caused by a mutant in ALADIN (for alacrima-achalasia-adrenal insufficiency neurologic disorder) [79], the Cockayne syndrome caused by a mutation in the cockayne syndrome A or B gene (CSA or CSB) [106,218] and X-linked late-onset sensorineural deafness, which is caused by a deletion in the transducin (beta)-like 1 gene (TBL1) [13,75]. Unfortunately, no (general) biological function for WD-repeats connected with human diseases has been found.

### *1.11 DM1 Mouse models*

Several groups have produced a number of different transgenic DM1 mouse models. In these studies they developed either transgenics to test hypotheses regarding DM1 disease pathology or to study the mechanisms underlying DNA instability (reviewed by Wansink and Wieringa 2003, *in press*).
Two groups, independent of each other, developed DMPK knockout mice with structurally similar gene lesions [100,180], resulting in complete absence of the DMPK protein. Only mild myopathy in neck and head were shown in one mouse model [100] while a late onset progressive skeletal myopathy and atrioventricular conduction abnormalities were reported for the other knockout mouse model [16,180]. Transgenic mice overexpressing the DMPK gene were also examined [100]. During breeding an increased prenatal and/or neonatal mortality and a high illness in pregnant and lactating females was observed, suggesting an effect of high DMPK levels during pregnancy and development. However, no abnormal features were observed in various tissues examined, except for a mild myopathy that was detected in heart muscle [100]. As mentioned before one of the hypothesis is that the expanded repeat could also affect other genes than the DMPK gene. Therefore, two Six5 knockout mouse model were created independently [113,187]. Both Six5 knockout mouse models showed an ocular cataract, a symptom (although clinical not entirely similar) also seen in DM1 patients [113,175,187].
Other research strategies were focussed on obtaining a better understanding of the mechanisms behind the instability of the $(CTG)_n$-repeat proper, as well as on the possible consequences of its expression in different tissues. Monckton and co-workers generated mouse models with exon 15 of the human DMPK gene, spanning a $(CTG)_{162}$ repeat randomly integrated [153]. All mouse models showed intergenerational and somatic instability of the repeat. The somatic instability seemed to be dependent on the site of integration [52,153]. Gourdon's group generated a series of different mouse lines carrying randomly integrated, large (45 kb) human genomic DNA inserts spanning the entire *DMWD-DMPK-SIX5* area with repeats of either 20, 55 or > 300 CTG-triplets (named DM20, DM55, and DM300 respectively [69,194]. In the DM20 lines no intergenerational length changes or somatic instability was detected. But a moderate germ line instability and somatic instability was observed for the DM55 lineages and a dramatically increased germ line instability as well as somatic instability was seen for the DM300 lines. This suggests that the size of the repeat is directly correlated to the rate of instability of the repeat (intergenerational and somatic).

Somatic instability was clearly age related and became prominent 6 to 9 months after birth [128,194]. The DM300 mouse lines displayed also a great number of typical DM1 disease features [193].
Again another series of transgenic mice with a $(CTG)_5$-repeat or a $(CTG)_{250}$-repeat spliced into the middle of the last exon of the human skeletal muscle actin gene (HSA) was generated with the aim to better understand the toxic RNA effects. These transgenes were designated $HSA^{SR}$ and $HSA^{LR}$, respectively, and served to monitor the consequences of expression of an untranslated expanded $(CUG)_n$-repeat when placed in a "DM1-unrelated" transcript [139]. $HSA^{LR}$ mice displayed many of the characteristic skeletal muscle symptoms of DM1 patients, supporting the view that presence of long $(CUG)_n$ repeats in RNA are both necessary and sufficient for development of aspects of DM1 pathology [139].

The last $(CTG)_n$ containing mice models discussed here are transgenic mouse models in which the entire 3'-DMPK-gene region was replaced by the exact cognate human segment, with either a normal $(CTG)_{11}$-repeat or an instable $(CTG)_{84}$-repeat [221]. The $(CTG)_{11}$-repeat was completely stable, while the $(CTG)_{84}$-repeat slowly expanded during intergenerational segregation and showed clear somatic instability, which was progressive upon aging [221].
Evidence that the mismatch-repair (MMR) machinery might be involved in trinucleotide instability came from the group of Manley who showed that mismatch repair enzyme Msh2 had a stabilizing effect on the trinucleotide repeat that caused Huntington disease [140]. Van den Broek and co-workers also examined the involvement of the mismatch repair enzymes with the trinucleotide instability of DM. When the $(CTG)_{84}$-repeat was introduced into an *Msh3*$^{-/-}$ background, somatic repeat instability was completely blocked, whereas a *Msh6* deficiency had a stimulating effect on the somatic mutation frequency, suggesting the involvement of proteins from the DNA repair and recombination machinery [221].

### *1.12 Outline of this thesis*

The main goal of the research reported in this thesis was elucidate the biological function of the DMWD protein and the possible role of the DMWD gene in the DM1 disease manifestation.
To this end we analyzed the localization of the protein in the two main expressing tissues namely brain (chapter 2) and testis (chapter 3), we searched for partner proteins of DMWD protein (chapter 4), examined the protein sequence for biochemical properties and homology to other proteins (chapter 5) and analyzed the homologous DMWD gene in *C. elegans* (chapter 6).

# Chapter 2

## The DMWD protein from the Myotonic Dystrophy (DM1) gene region is developmentally regulated and most prominently present in synapse-dense brain areas

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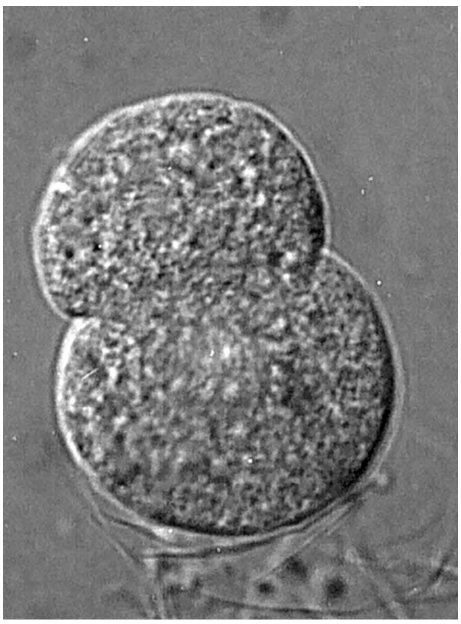

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**Summary**

The *DMWD* gene is located in the myotonic dystrophy (DM1) gene cluster on 19q, just upstream of the *DMPK* gene. RNA and protein products of this gene are ubiquitously expressed in all adult tissues, but occur most abundant in testis and brain. Altered expression of DMWD mRNA in DM1 patients has been observed, suggesting a role of the *DMWD* gene products in disease manifestation. Here we focussed on DMWD expression in mouse brain and followed mRNA and protein levels and (intra)cellular location in developing brain *in vivo* as well as in differentiating neuronal cell-cultures *in vitro*. In the interval between postnatal days P7 to P21, the steady-state level of DMWD mRNA remained constant, whereas the DMWD protein (doublet of 70kDa) level gradually increased during the same period. The DMWD protein was expressed throughout the brain, at a low level in glial cells, more prominently in neurons and specifically in the neuropil of brain areas with a high density of synaptic connections. Intracellular, DMWD was dispersed in a punctuate fashion throughout the neural cell body, the nucleus and the dendrites with their synapses, but was excluded from axons. Based on these findings and on new literature data concerning the role of *DMWD* homologs in lower eukaryotes, we discuss the possible role of DMWD in the brain-related symptoms seen in DM1 patients.

## Introduction

Myotonic dystrophy (DM1) is the most common form of muscular dystrophy in adults with an incidence of 1: 8000 [83]. DM1 is caused by an unstable $(CTG)_n$-repeat located in the 3`-UTR of the Myotonic Dystrophy Protein Kinase gene (*DMPK*) [24,56,137]. The molecular basis by which the expanded repeat causes Myotonic Dystrophy is not understood, but based on different studies there is now consensus that the expanded $(CTG)_n$-repeat – when expressed in DMPK mRNA - may confer a toxic gain-of-function effect on the expression of mRNAs from various other genes. Strong evidence points to the involvement of (pre-)mRNA binding proteins with a role in splicing and/or nuclear export[170,214,215]. The $(CTG)_n$-repeat may also cause a topologic chromatin distortion, which might result in altered transcription of either one of three genes in the DM1 locus, *DMPK*, *Six5* (formerly called *DMAHP* [21]) and/or *DMWD* (formerly known as *DMR-N9* in mouse and as *gene 59* in human). The *DMWD* gene may be involved in the manifestation of DM1 because it is located just upstream of the *DMPK* gene and it is prominently expressed in tissues affected in DM1 patients, e.g. brain and testis, in which the *DMPK* gene is hardly expressed. The gene's protein product has a proline rich N-terminal part and an internal segment with four WD-repeat units. Other WD proteins usually contain 4 - 8 WD-repeats, which fold into a twisted propeller like structure with as many blades as WD-repeat units [61,199]. Remarkably, until now there is no obvious functional relationship in biological function between different WD-proteins, although different sub-families can be distinguished based on the classes of proteins they interact with or the domains found in combination with the WD-repeats [31,158,199].
Here we follow up on the hypothetical possibility that DMWD expression distortion in brain of DM1 patients may be involved in DM1 typical features like the mental retardation, enlarged ventricles and cerebral white matter lesions, sleep disorders and behavioural changes [83]. Study of DMWD expression in brain in vivo and in cultured neurons in vitro revealed that DMWD mRNA and protein levels are under differential developmental control. Protein expression is temporally regulated with a specific spatial distribution in neurons in brain in areas that contained many synaptic endpoints. We discuss our findings with respect to recent findings on DMWD function in lower eucaryotes and speculate about the possible pathobiological role of DMWD in brain of DM1 patients.

## Materials and methods

*DMWD antibody generation (abB13and abH84)*
AbB13: A partial DMWD construct (amino acid 114 - 650) was created by the use of PCR with primer pat1 5´-GGA GAA TTC GAG CTT TAT TTC TAC CCC-3´ (nucleotide position 343 to 361) and pat2 5´-AGC GAA TTC TTA TCA CAC CAC AGT GCC-3´ (nucleotide position 1935 to 1953) on mouse cDNA clone 9.1 [99] (accession numberZ38011-Z38013 and Z38015). This PCR product was digested with EcoRI and ligated in frame into the EcoRI site of the pAr (delta EcoRI)(59/60) expression vector [18,209]. Next, the pArDMWD vector was introduced into *E.coli* BL21(DE3)pLysS (Stratagene, California, USA) and induced with 0.33 mM IPTG (isopropylthio-β-D-galactosidase). The integrity of the induced protein (~60kDa) was checked by analysing a small protein sample on a 10% w/v SDS-polyacrylamide gel and the remainder was run on a large 10% w/v SDS-polyacrylamide gel for preparative purification. Protein with the correct size was excised from the gel and the protein was retrieved by electro elution in a Biotrap BT100 apparatus (Schleicher & Schuell) according to manufacturer's protocols.

The protein concentration was determined by the Lowry procedure according to Peterson [169] and protein purity was checked again by analysis on a 10% w/v SDS-polyacrylamide gel. A rabbit was immunized once with 400 µg of the purified DMWD protein in a 1:1 v/v ratio with incomplete Freund's adjuvant and three times with 300µg purified DMWD protein, also in a 1:1 ratio. Two months later, after reactivity of the serum was checked with a Western blot on which the DMWD protein was blotted the rabbit was bled and the serum collected and affinity- purified over a column in which the truncated DMWD protein was bound to a matrix (Affi-gel10, Bio-Rad) in 0.1 M MOPS buffer according to the manufacturer's protocols. Purified antibody (designated abB13) was kept at –20 and –80°C.

AbH84: DMWDfl (full-length) [98] (accession numberZ38011-Z38013 and Z38015) was cloned in the EcoR1 site of pFastBac$^{TM}$HTa and pFastBac$^{TM}$Dual vector (Life Technologies). The insert orientation in the plasmid was tested with restriction enzyme digestion and DNA sequence analysis. Sf9 cells were infected with either one of the correct plasmid vectors (pFastBac$^{TM}$HTaDMWDfl or pFastBac$^{TM}$DualDMWDfl) and presence of DMWDfl proteins in total cell-lysates was tested by Western blot analysis (detection with abB13). Thereafter DMWDfl protein was purified on a large preparative SDS-PAA gel and retrieved by electro-elution, the protein concentration was determined and a rabbit was immunized with the purified DMWD protein as detailed above. Two months later the rabbit was bled, serum was collected and purified as described for the abB13. Purified antibody (designated abH84) was kept at –20 and –80°C.

*Protein analysis using Western blotting*

Total brain tissue was collected from C57BL/6 mice at 1, 4, 7, 10, 15, 21 and 28 days of age (day 1 = day of birth), snap-frozen in liquid nitrogen, thawed and homogenized on ice in an appropriate volume of buffer (10 mM Tris pH 8, protease inhibitor cocktail (Boehringer Mannheim) and PMSF). Protein concentrations in the extracts were determined by the Lowry procedure [169] and samples with an equivalent of 45 µg of total protein were analyzed by electrophoresis on a 10% w/v SDS-polyacrylamide gel and western blotting. Therefore, proteins were transferred onto nitrocellulose membranes (Schleicher & Schuell, Keene, NH). Membranes were blocked in 5% skimmed milk in Phosphate Buffered Saline (PBS) with 0.05% Tween-20 (PBST) for minimal an hour at RT. Antibodies were diluted in fresh blocking solution and incubated o/n at 4°C on a rotating platform (abB13 1:5000, abH84 1:5000). The blots were washed with PBST and incubated with peroxidase-conjugated goat anti-rabbit immunoglobins (Pierce, Rockford, IL) for minimal an hour at RT on a rotating platform, washed again with PBST and PBS and developed using a chemiluminescence Western blotting reagent.

*Immunohistochemical localization of DMWD protein*

The entire brains from 5 weeks old C57/BL6 mice were removed and immediately frozen in liquid nitrogen. Brain sections (8-10 µm) were sliced on the cryostat (Leica), mounted on silane-coated slides, dried and kept at –80°C until further use.

At day of use slides were placed under a ventilator at RT and dried for at least one hour. They were fixed for 5 min in 3% paraformaldehyde in PBS, washed with PBSBG (0.15% BSA, 0.15% glycine in PBS) and incubated with 1.5% $H_2O_2$ in PBSBG for 20 min. at RT. Again they were washed with PBSBG and pre-incubated, for at least 30 min in a humid chamber, with 2% Normal Donkey Serum (NDS) in PBSBG. The antibody incubation was done o/n at RT in a humid chamber with abB13 1:200 and abH84 1:200 in PBSBG with 1% NDS.

Slides were washed with PBSBG and incubated with biotin-labelled donkey anti rabbit (1:250: Jackson Immuno Research Laboratories, Inc, West Grove, USA) in PBSBG for 1 hour at RT, then washed, incubated with the ABC solution (Vectastain) for 30 min at RT, and washed with PBST and PBS. Finally, the protein was detected with the AEC solution (1 ml of 1 N,N-dimethylformamide (4 mg/ml; BDH, Germany), 14 ml 0.1 M Acetate buffer pH 4.9, 15 µl $H_2O_2$ (30%)). The colouring reaction was stopped by washing with water, and the slides were embedded in Kaisers gelatine (BDH, Germany).
Sections were analyzed using the light-microscope and cell-layers or cell groups were nominated according to the mouse in stereotaxic coordinates brain atlas by K.J. Franklin and G. Paxinos (1997) [53]. The different brain areas used for DMWD analysis:
Olfactory bulb: +4.28, +3.56 and +2.58 mm from Bregma. Forebrain: +2.58, +1.70 and +0.62 mm from Bregma. Thalamus: +0.50, +0.14, -0.10 and –1.70 mm from Bregma. Hypothalamus: -0.70, -1.70 and -2.46 mm from Bregma. Hippocampus: -1.70 and -2.46 mm from Bregma. Mid/hind brain -4.60 mm from Bregma. Cerebellum: -4.60, -5.34, -5.52 and -6.48 mm from Bregma. All slides were scored/interpreted by two independent observers. Gradual score from -: no DMWD staining, +: weak DMWD staining, ++: clear DMWD staining, +++: strong DMWD staining.

*RNA analysis*
Total brain tissue was collected from C57/BL6 mice at 1, 4, 7, 10, 15, 21 and 28 days of age, snap-frozen in liquid nitrogen, thawed and homogenized on ice in an appropriate volume (for every 100 mg tissue, 2 ml of RNAzol) of RNAzol™B (Campro Scientific). After thorough homogenization, 1/10 volume of chloroform was added and mixed with the RNAzol solution. This was centrifuged at 13000 rpm for 20 min. at 4°C and the fraction containing the RNA was precipitated on ice in a new tube with one volume of isopropanol. Again the mixture was centrifuged and the pellet was washed twice with 75% ethanol, dried and kept under 70% ethanol in NSE (50 Mm NaAc, 0.2% SDS, 2 mM EDTA) at –20°C until further use. RNA concentrations were determined (from $E_{260\ nm}$/ $E_{280\ nm}$ readings and a 1% agarose test gel).
For each sample 10 µg of total RNA was analyzed by Northern analysis after o/n electrophoresis on a 1% agarose/formaldehyde/ MOPS gel. After a picture of the ethidium bromide stained gel was taken, the RNA was transferred to a nitrocellulose membrane (Schleicher and Schuell) and cross-linked by UV radiation. Northern blots were hybridized o/n at 65°C in a rotating chamber with a randomly labelled ($\alpha$-$^{32}$P-CTP, Amersham) 2.2 kb EcoRI fragment from DMWDfl cDNA and visualized by exposure to Kodak X-OMAT film (18 to 72 hours).

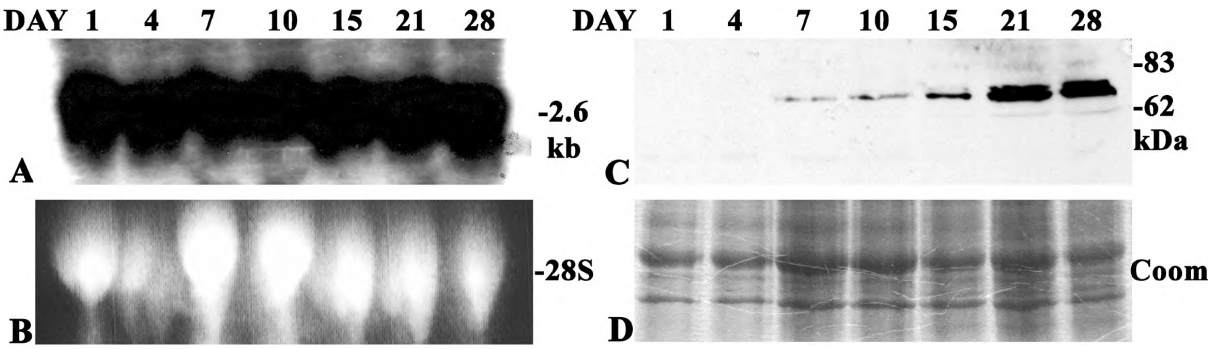
*Primary neuron culture*
Neurons were cultured as described in de Hoop et al. with some adaptations [90]. Briefly, neurons were co-cultured with astrocytes to provide the right survival factors for the neurons. Hippocampi of 16.5 dpc embryos (E 16.5) were isolated, dissociated with trypsin and plated on sterile, poly-l-lysine coated coverslips in 24-wells plates. They were allowed to attach for 3-4 hours in DMEM/5%FCS at 37°C and then placed upside down on top of astrocytes (30-50% confluent) that were already cultured in the 24-wells plates. The cell cultures were maintained in NBM ((50 ml NBM (Gibco) with gentamicin (end concentration 0.5 mg/ml), 100 µl glutamine (200 mM) and 1ml B27 (Gibco)) at 37°C in a humidified chamber and half of the medium was replaced with new medium every 7 days. After culturing for a defined period of time, coverslips were washed, cells were directly dissolved in 2 X SDS sample buffer (200 mM Tris-HCl, pH 6.8, 400 mM dithiothreitol, 8% SDS, 0.4% bromophenol blue, 40% glycerol) and analyzed for DMWD expression on a Western blot or cells were washed with PBS and fixed with 3% paraformaldehyde in PBS for max. 5 min.

Coverslips were washed again with PBS, permeabilized with 0.5% NP-40 in PBS for 5 min. and incubated with 100mM glycine in PBS. After this, slides with cells were pre-incubated with 1% NGS in PBST for 30 min. and subsequently incubated for at least two hours with antibodies in a humid chamber (abH84 1:200, abB13 1:200 and NF200 1: 200). Then the slides were washed, incubated with the secondary antibody (GAR or GAM, both Jackson Immuno Research Laboratories, Inc, USA) for 30 min. in the humid chamber, washed again, embedded in Mowiol (Sigma Chemical) and analyzed with a confocal laser-scanning microscope (MRC1000, Bio-Rad). To block the antibody, we pre-incubated the antibody solution with (1:1) SF9 cell-lysate containing DMWDfl protein (total protein concentration approximately 40 µg/µl) on ice for at least an hour. Just prior to the antibody incubation, we spun the antibody/SF9 solution and used the supernatant to perform the antibody incubation with the cultured neurons as described above.

## Results

*Postnatal DMWD expression*

To obtain clues for the biological significance of DMWD gene in brain, we examined its postnatal expression and product-distribution profile at the RNA and protein level. For this purpose, we first isolated RNA from postnatal developing brain at different time points after birth (period between P1 {day of birth} and P28) and analyzed these samples for DMWD mRNA expression on Northern blots (Fig.1A). For analysis of DMWD protein expression levels, total brain lysates were isolated at the same time points as for the RNA samples and subjected to analysis on Western blots (Fig. 1C). DMWD mRNA was clearly present at all time points and its expression level did not change relative to the total RNA content (18S+ 28S rRNA) during this period (Fig. 1A/B). In postnatal developing brain, DMWD protein (visible as a 70 kDa doublet; predicted size 69 kDa) appeared at day P7 and was not fully present until day P21 (Fig.1C). The DMWD signals on these blots were fully blocked in presence of native DMWD produced in the baculovirus-Sf9 cell production system (not shown), and can therefore be considered entirely DMWD-specific. From day P21 onwards and during further maturation and aging, the level of DMWD in brain remained constant (not shown). Thus, although the level of DMWD mRNA was approximately constant during the first four weeks of life, DMWD protein levels increased dramatically during this same period. This suggests that DMWD in brain serves a specialized function, which is strictly regulated and apparently not required during the initial phases of postnatal brain differentiation and maturation.



*DMWD localization*

Next, we examined adult mice brain (36 days old) with two different DMWD antibodies (abB13 and abH84, prepared against partial and full-length DMWD protein, respectively) for the localization of the DMWD protein (Table 1 and Fig. 2). Both antibodies showed staining in the same areas and same cells, although in general the signal with abB13 was stronger than abH84, with two exceptions i) the hypothalamic area where abH84 gave a stronger signal than abB13 and ii) the purkinje cells which did not stain with abB13 but sometimes gave a signal with the abH84. Because these purkinje cells were not positive with the abB13 and stained only weakly with the abH84, we scored them as DMWD negative (Table 1).

We checked for specificity of both antibodies by blocking tissues sections (and Western blots) with DMWD infected Sf9 cell-lysates or mock infected Sf9 cell-lysates. None of the sections (or Western blots) blocked with baculovirus produced DMWD in Sf9 cell-lysate showed any DMWD signal, whereas the sections blocked with the mock infected lysate showed clear DMWD staining (not shown for brain sections; but see neuron specific staining in Fig.5).
An overview of the DMWD protein expression distribution as assessed by quantitative examination of serial coronal sections of the entire mouse brain (anterior to posterior, starting with the olfactory bulb) is shown in Table 1. Throughout the brain, only few regions occurred that lacked DMWD staining entirely. DMWD protein was most prominently expressed in areas where many synaptic end points were coming together, like the glomeruli of the olfactory bulb and the cerebellum (Fig. 2).

*Olfactory bulb*

In the olfactory bulb, a clear pattern of DMWD staining was seen coinciding with the various cell-layers of which the olfactory bulb consisted (Table 1, Fig.2A1). The myelin tracks in the centre of one olfactory bulb half as well as the mitral cell-layer further to the outside, were DMWD negative. The internal plexiform layer showed DMWD signal but less than the anterior olfactory nucleus (Fig. 2A2) and the granular layer. The granular layer consists of granule cells, which were DMWD negative, and the surrounding areas, which were DMWD positive. The external plexiform layer and the glomerular layer were both darkly stained with the DMWD antibodies. Within the glomerular layer, the glomeruli were strongly stained with both the DMWD antisera (Fig. 2A3).

*Forebrain*

Sections of the forebrain showed a more moderate DMWD expression than the olfactory bulb. DMWD protein was detected in cortex layers II-VI, in the cortex neuropil, the accumbens neuropil and nuclei, the striatum cells and neuropil, and the bed nucleus stria terminalis. Rather weak staining was seen in the piriform cortex and the amygdaloid area. Although the cortex layers II-VI showed DMWD expression, no antibody staining was detected in cortex layer I, nor in the globus pallidus.

**Fig. 1** (previous page). DMWD expression levels (RNA and protein) in postnatal developing brain. Panel A: Northern blot analysis showing DMWD RNA expression levels; RNA was isolated from brain at day 1, 4, 7, 10, 15, 21 and 28 days after birth. Panel B: 28 S RNA of the gel used for Northern blot to show (approximate) equal loading. Panel C: Western blot analysis showing DMWD protein expression, total brain lysates were isolated from mice at day 1 until day 28 after birth. Panel D: Coomassie staining of replica protein gel. Note: day 1 is day of birth.

**Table 1.** DMWD protein expression in various areas of the mouse brain.

| | | | |
|---|---|---|---|
| *Olfactory bulb* | | *Hypothalamus* | |
| - Myeline tracks | - | - Anterior hypothalamic nucleus | + |
| - Granular layer [#] | ++ | - Arcuate hypothalamic nucleus | + |
| * Granular cells | - | - Dorsomedial hypothalamic nucleus | + |
| * Glomeruli | ++ | - Ventromedial hypothalamic nucleus | + |
| - Internal plexiform layer | +/- | | |
| - Mitral cell-layer | - | *Hippocampus* | |
| - External plexiform layer | +++ | - Mossy fibers (terminal fields) | |
| - Glomerular layer | +++ | * stratum oriens | ++ |
| - Anterior olfactory nucleus | ++ | * stratum lucidum | +++ |
| | | * stratum radiatum | ++ |
| *Forebrain* | | * Dentate gyrus hilus | +++ |
| - Cortex layer I | - | * Supra granular layer | ++ |
| - Cortex layer II-VI | ++ | - Granular cell-layer Dentate gyrus | - |
| - Cortex neuropil | + | - Pyramidal cell-layer CA 1, 2 and 3 | - |
| - Accumbens nucleus | + | | |
| - Accumbens neuropil | ++ | *Cortex Mid/hind brain* | |
| - Striatum cells | ++ | - Dorsal neuropil | +/- |
| - Striatum neuropil | ++ | - Ventral neuropil | + |
| - Globus pallidus | - | | |
| - Bed nucleus stria terminalis | ++ | | |
| - Piriform cortex | +/- | *Cerebellum* | |
| - Amygdaloid area | +/- | - Myeline tracks | - |
| | | - Granular layer [#] | +++ |
| *Thalamus* | | * Granular cells | - |
| - Geniculate nucleus | ++ | * Glomeruli | +++ |
| - Posterior thalamic nucleus | +/- | - Purkinje cells | - |
| - Ventral posteromedial thalamic nucleus | ++ | - Molecular layer | ++ |
| - Zona incerta | + | | |
| - Substantia nigra | + | | |
| - Medial mammillary nucleus | ++ | | |

DMWD protein distribution profile in the mouse brain. Sections from the different brain areas indicated were immuno-stained with antibody abB13 or abH84, and distribution patterns was analyzed using the light-microscope. The intensity of immuno-staining was scored, -: no DMWD staining, +/-: very weak DMWD staining, +: weak DMWD staining, ++: clear DMWD staining, +++: strong DMWD staining.
[#]: cell-layer is divided in cells and surrounding area (glomeruli).

*Thalamus*

In the thalamic area, the geniculate nucleus, the ventral posteromedial thalamic nucleus and the medial mammillary nucleus were stronger stained than the zona incerta and the substantia nigra, but they all expressed DMWD. The posterior thalamic nucleus showed very little DMWD reactivity.

*Hypothalamus*

The hypothalamic area showed an even distribution of DMWD, all hypothalamic nuclei displayed a low level of DMWD expression.

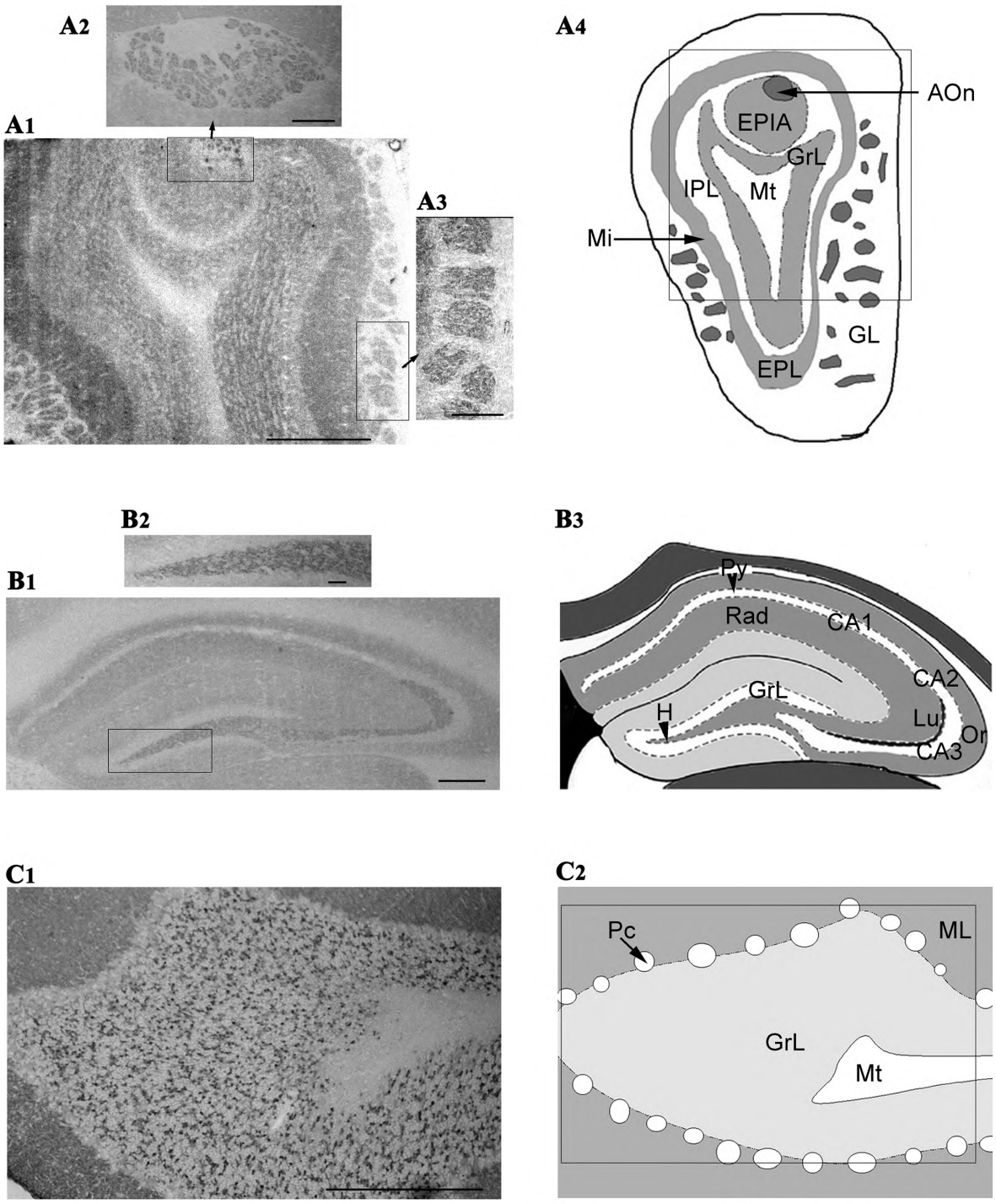



**Fig. 2**. Distribution and intensity of DMWD antiserum staining throughout different mouse brain areas. Panel A1: olfactory bulb overview. Panel A2: enlargement of the anterior olfactory nucleus. Panel A3: enlargement of glomeruli. Panel A4: schematic overview of olfactory bulb. Panel B1: overview of hippocampus. Panel B2: enlargement of dentate gyrus hilus. Panel B3: schematic drawing of hippocampus. Panel C1: part of cerebellum. Panel C2: schematic drawing of cerebellum. Panel A4: AOn: anterior olfactory nucleus, EPL: external plexiform layer, EPIA: external plexiform layer anterior, GL: Glomeruli layer, GrL: Granular layer, IPL: Internal plexiform layer, Mi: Mitral cell-layer, Mt: Myeline tracks. Bar in A1, A2, A3 represents 625 μm. Panel B3: CA1: CA1 field of hippocampus, CA2: CA2 field of hippocampus, CA3: CA3 field of hippocampus, GrL: granular layer, H: dentate gyrus hilus, Lu: stratum lucidum, Or: Stratum oriens, Py: pyramidal cell-layer hippocampus, Rad: stratum radiatum. Bar in B1, B2 represents 150 μm. Panel C2: GrL: granular layer, ML: molecular layer, Mt: Myeline tracks, Pc: Purkinje cells. Bar in C1 represents 375 μm.

*Hippocampus*
In the hippocampus area, we detected strong DMWD staining in the mossy fibre projection fields i.e.: stratum oriens, stratum lucidum, stratum radiatum and in the dentate gyrus hilus, and supra granular layer (Fig. 2B1). However, the pyramidal cell-layers (CA 1, 2 and 3) and the dentate gyrus granular cell-layer completely lacked DMWD staining.

*Cortex Mid/hind brain*
The ventral neuropil in the mid/hind brain showed a stronger immunoreactivity than the dorsal neuropil in this same region, but both expressed the DMWD protein.

*Cerebellum*
The cerebellum showed a clear pattern i.e., the myelin tracks, rich in axons, and the granule cells (in the granular layer) were DMWD negative, but the molecular layer and the glomeruli in the granular cell-layer, were DMWD positive (Fig. 2C1). The purkinje cells located in between the molecular and granular layers were apparently devoid of DMWD.

*DMWD expression in the eye*
To test if DMWD is indeed present in areas with many synaptic connections, we turned to the retina of the eye as a tissue, which is particularly rich in these structures. We analyzed both rat and mouse retina. The retina in rats and mice gave similar results; the pictures shown are taken from the rat eye as these gave a better overview of the different layers. Results of these analyzes are shown in Fig. 3, next to a schematic picture and histological overview of retina structure [104], which were added for clarity.

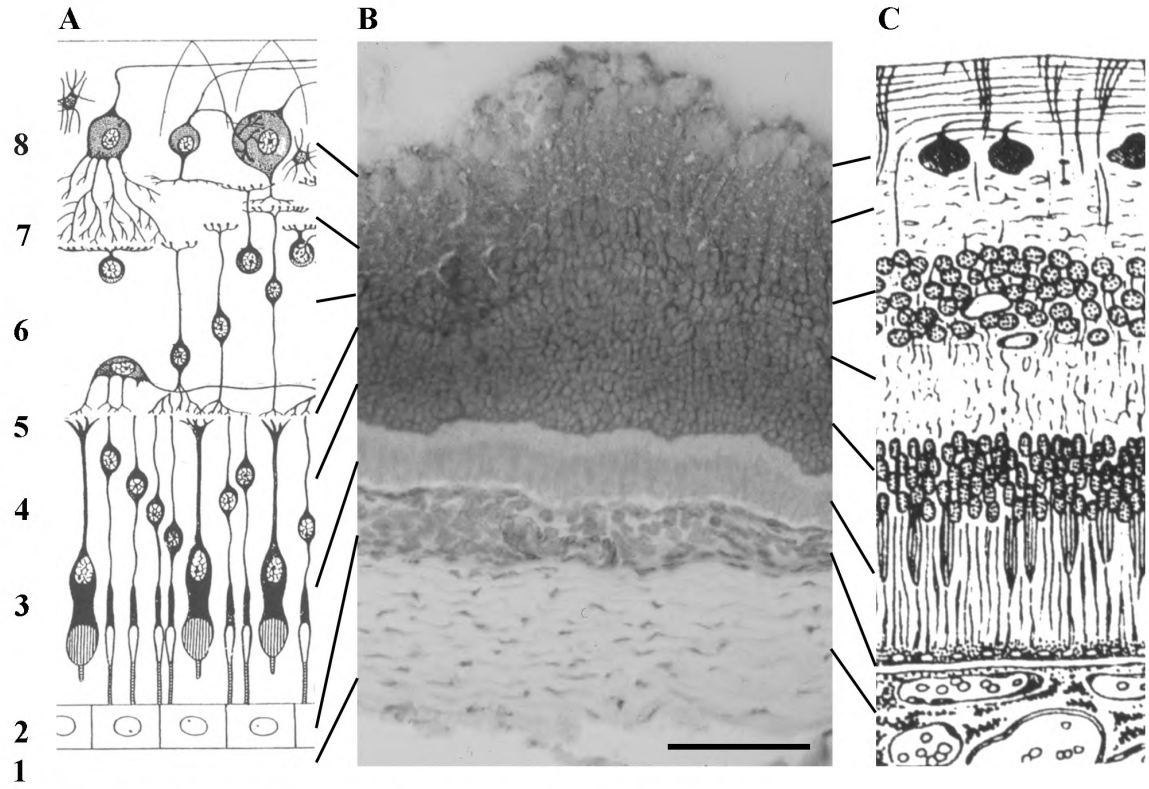



**Fig. 3.** Immunohistological distribution of DMWD in the retina of the rat.
Panel A: schematic structure of the retina [104]. Panel B: DMWD staining of the retina, from rat eye. Panel C: General histological appearance of the retina [104]. Layers: 1: choroidocapillary lamina, 2: pigmented epithelium, 3: rods and cones, 4: external granular layer, 5: external plexiform layer, 6: internal granular layer, 7: internal plexiform layer, 8: ganglionic layer. Bar in B represents 75 μm.

In the retina, DMWD antibody staining was strongest in the areas that contained the highest density of synaptic junctions (dark grey represents DMWD staining), such as the internal plexiform layer (compare Fig. 3B to layer 7 Fig. 3A). This layer consists of dendrites of the large multi polar nerve cells that form synapses with the amacrine cells (cells with the many short dendrites) and axons of the bipolar neurons.
Also the external plexiform layer (Fig. 3A, layer 5), although much narrower than the internal plexiform layer, stained clearly DMWD positive. In both the internal (layer 6) and the external granular layer (layer 4) we saw that the granule cells themselves were not stained, whereas some dendrites in between the granule cells were. Also the large multipolar nerve cells expressed the DMWD protein, not only in their dendrites, but also in the cell body. The different layers that were not stained with DMWD antibody (layers 1, 2 and 3) do not contain any dendrites. This latter finding is consistent with our observation that DMWD was also mainly expressed in dendrites and areas with many synaptic endings.

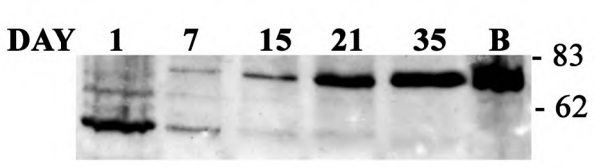



**Fig. 4.** Western analysis of extracts from primary neuron cultures, detected with abB13. Day 1: day cells were spread, Day 7 till 35: days after spreading, B: total brain isolation of adult mouse, as a control. The band shown in the lane of day 1 is of unknown origin.

*DMWD expression in cultured neurons*
To mimic the situation in terminally differentiated neurons in brain, we kept primary hippocampal neurons for several days to weeks in *in vitro* culture. Upon prolonged cultivation, cells formed more dendrites and synapses, resulting in the appearance of an elaborate neuronal network (microscopic analysis not shown).
Western blot analysis of lysates from cultured primary hippocampal neurons prepared from brain of 16.5 dpc old mouse embryos and kept for 1 to 35 days in culture revealed that DMWD expression increased with progressing differentiation in culture (Fig. 4). At day 1, the 70 kDa DMWD protein was not detectable, but at day 7, DMWD protein began to appear but the doublet was not fully present until day 21. Thus, the graded increase in DMWD level that we saw in brain from mice of P1 - P28 after birth (Fig. 1) was mimicked in cultured neuronal cells.

Finally, to examine the intra-cellular localization of DMWD in these neurons, we performed immunostainings with the abB13 antibody (Fig. 5). The neurons were always cultured as a mixture of neurons (NF200, high-molecular-weight neurofilament, positive) and glia (GFAP-positive) cells. The glia cells in these cultures were not analyzed in detail in this study although they consistently showed light DMWD staining. Mature neurons (NF200 positive) displayed a much more intense staining, distributed in a punctuate pattern across different regions of the cell body, the nucleus and in the dendrites (Fig. 5). Strikingly, DMWD staining was fully absent in the axons of the neurons. Again this staining was DMWD positive as antibody reactivity was fully blocked in presence of native DMWD in baculovirus infected Sf9 cell-lysates (Fig. 5E) but not with mock infected Sf9 cell-lysates (Fig. 5C and D).

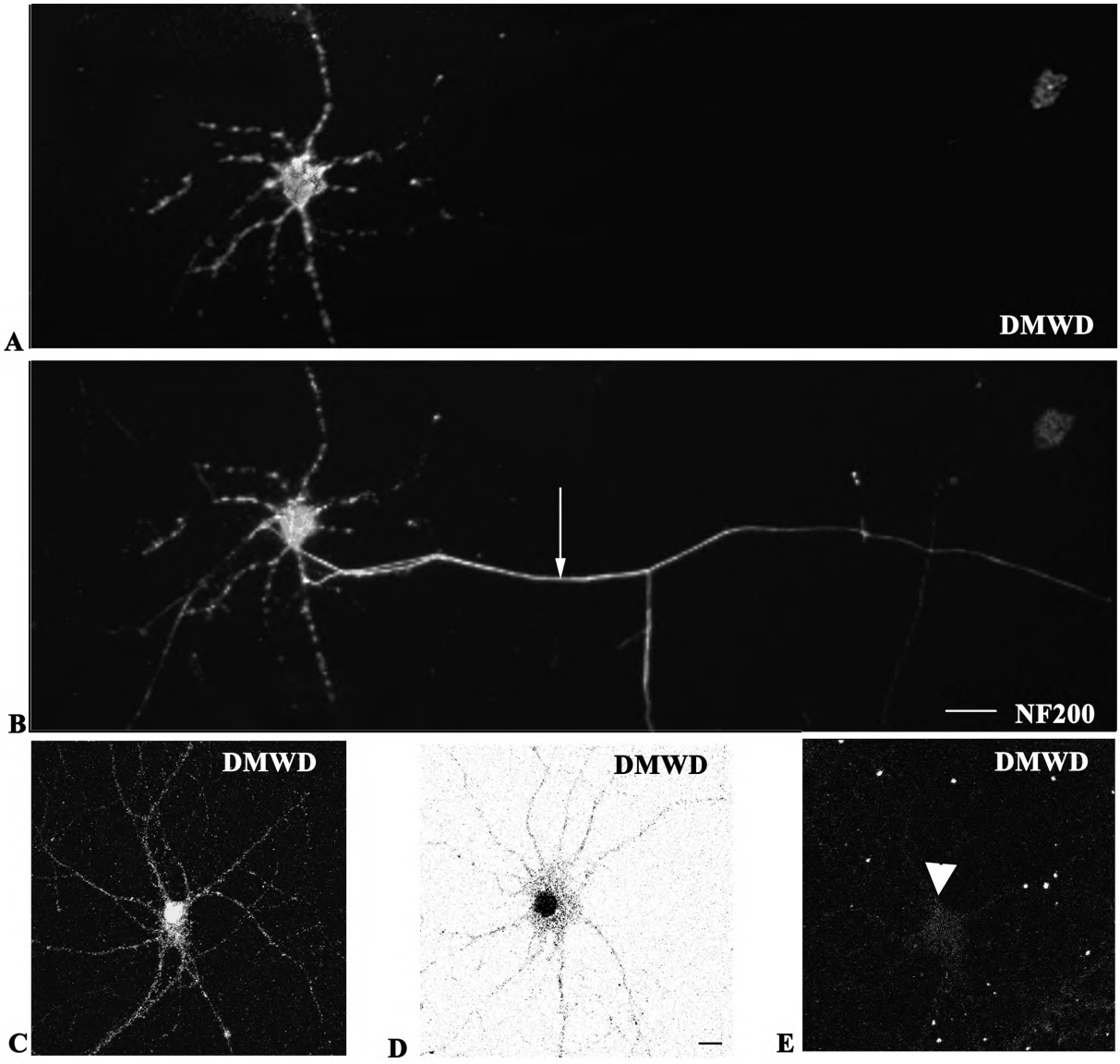



**Fig. 5.** Intracellular DMWD distribution in differentiated primary hippocampal neurons in in vitro culture.
Panel A: neuron stained with abB13. Note that DMWD protein is dispersed across the cell body and the dendrites, but not present in the axon. Panel B: the same neuron stained with a neuron marker, NF200, showing the neuron with its cell body, the dendrites and the axon (indicated by arrow). Panel C and Panel D (inversed image): two different neurons stained with abB13, after pre-incubation with mock infected Sf9 cell-lysate, which does not block reactivity of the antibody. Note again the punctuated DMWD staining in the cell body, the nucleus and the dendrites of the neurons. Panel E: neuron stained abB13, after previous incubation with native DMWD produced in baculovirus infected Sf9 cell-lysate (see M&M). DMWD staining has fully disappeared. The arrowhead indicates the position of the cell body of the neuron. Bar represents 10 μm.

## Discussion

In this study, we have shown that DMWD RNA levels remain constant during postnatal development of the mouse brain, whereas DMWD protein levels start to increase from day 7. In mature animals DMWD protein was expressed throughout the brain, but occurred most prominent in areas with many synaptic connections. Also, in cultured cells *in vitro* the level of DMWD expression increased with time and was highest when neurons became fully developed and possessed extended dendritic projections. These results and the possible implications for DM1 patients are discussed.

*DMWD RNA and protein levels*

During postnatal development (the first 28 days after birth) of the mouse brain, DMWD RNA did not change, whereas DMWD protein levels increased from day 7 onwards. The fact that there is no linear relationship between mRNA and protein levels suggests that *DMWD* is either not translated or that the half-life of DMWD protein is strictly differentially regulated at different phases of development, and the protein is very labile when brain development and maturation are ongoing.

The faster migrating component of the DMWD protein-doublet was already detected at P7, clearly before the larger DMWD protein appeared at P21 in the mouse brain. We checked for alternative splicing of the DMWD RNA in brain, but could not detect any alternative spliced products (similar to that found for testis, unpublished observations). Therefore, we consider it most likely that the smaller DMWD protein is the primary gene product and that the larger DMWD protein evolved by modification. Whether this involves phosphorylation or methylation or any other type of modification that can determine protein fate remains to be resolved.

*DMWD postnatal expression in brain and DMWD localization in cultured neurons*

The expression of DMWD proteins increased in the first three weeks of postnatal brain development. This period coincides with the time that cerebellar neurons develop into mature cells and show maturation of all their dendrites and synapses [64]. The period between P21 and P28 corresponds with the peak of synapse formation in the rat CNS [11]. This study demonstrated also an increase in DMWD protein expression in *in vitro* cultured neurons, which enabled us to follow neuronal growth and development in a more simplified manner than in the three-dimensional context of the entire brain. Differentiation occurred predominantly during the first two weeks of culture and the neuronal phenotype observed highly resembled that of neurons *in vivo* [35]. Indeed, the longer the neurons were cultured, the more the neuronal network extended with its axons, dendrites and synapses. This study showed that the increase in DMWD protein was concomitant with the progressing differentiation of neurons and that the DMWD protein was fully expressed, after the third week, in both the cell body and the dendrites with their synapses of these cultured neurons. Also both protein forms could be detected. This suggests a temporal expression pattern for DMWD protein in neurons. Interestingly, the cultured neurons also showed a spatial restricted pattern, i.e. the axons did not contain any DMWD protein but the cell body, the nucleus and the dendrites (with the synapses) did. Consistent with this observation were the findings in the cerebellum and the olfactory bulb, where no DMWD protein could be detected in the myeline tracks, the site where the axonal processes join together, while DMWD expression was apparent in the adjacent cell-layers.

Similar temporal expression patterns in cultured neurons have been reported previously. Ferreira et al showed that after two weeks, synapsin IIa was increasingly expressed in cultured hippocampal neurons [50]. Synapsin IIa belongs to the synapsin family of synaptic vesicle proteins that play a crucial role in the regulation of neurotransmission and synaptogenesis [108], with each synapsin protein having its own temporal expression pattern and specialized function. Synapsin I and II are both needed for synapse formation and are located at the presynaptic nerve terminal in CNS neurons [50]. Since DMWD demonstrated a similar temporal expression pattern like synapsin IIa and because DMWD is -like synapsin- also expressed in synaptic areas, it is feasible that DMWD has a comparable specialized role in neuronal development and synaptogenesis.

*DMWD localization in adult mouse brain*

The DMWD protein was expressed in many areas throughout the whole adult mouse brain. The neuropil in all areas of the brain was stained lightly, but there were certain regions where DMWD antibodies stained much stronger: areas where synaptic connections were concentrated (Table 1, Fig. 2, and Fig. 3). These regions, like the glomeruli and granular layer in the olfactory bulb, the mossy fibre terminal fields in the hippocampus, and the glomeruli in the granular cell-layer of the cerebellum, are abundant in synaptic endpoints, and stained strongly for DMWD. To emphasize that DMWD was expressed in areas with a high concentration of synapses, we also examined DMWD expression in the different neuronal layers of the eye. We detected strong positive staining in the layers with many dendrites and a high number of synapses, specifically the internal plexiform layer and the external plexiform layer.

When taken together, we consider these findings support for our hypothesis that the DMWD protein serves a very specialized biological function in maturing neurons, and specifically in the dendrites with their synapses.

*DMWD and possible partner proteins in brain*

Recently it became clear that the *A. nidulans* homologue of DMWD: CreC interacts with and stabilizes the deubiquinating enzyme CreB [134,217]. This enzyme removes the ubiquitin moieties of CreA, a DNA-binding protein, resulting in high levels of CreA and thus repression of the gene(s) under its control [134]. In Drosophila it was found that synaptic growth and function was regulated by ubiquitination-dependent mechanisms, and the authors suggested that synaptic development might be controlled by the balance between positive and negative regulators of ubiquitination [44].

DMWD was detected in neurons in a time dependent pattern (comparable to that found for synapsin IIa [50]) and specifically in areas where many synapses were located. A homologue of CreB, the deubiquinating enzyme, is also found in human: UBH1 [80]. Although direct evidence is still lacking, by extrapolation of all this information to the situation for DMWD in mouse/human, it would be interesting to investigate possible interactions between DMWD and UBH1 in brain, more specifically in the synapses of different neurons and the role it might have in the transcriptional regulation of the processes involved in growth and maturation of dendritic synapses.

*DMWD and DM1*

Clinical manifestation in DM1 patients is highly variable and involves multiple systems and organs like muscle, brain, eyes, heart and testis [83]. The brain-related symptoms in DM1 are characterized by mental retardation, sleep disorders, behavioural changes and alterations in brain structure (ventricular enlargement and temporal lobe white matter changes) [43,84].

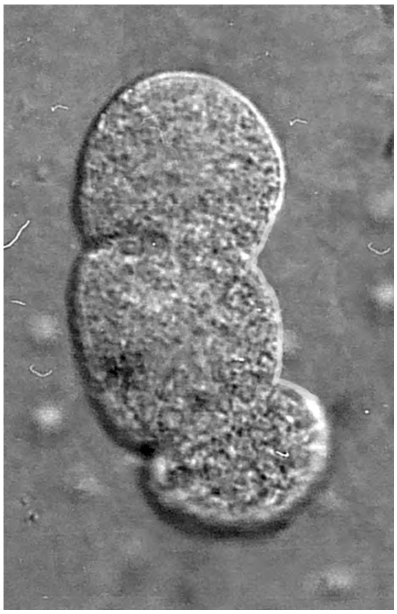
It is known that DM1 patients have cognitive defects as well, which are presumed to have their origin in the cerebral cortex. These cognitive defects are most prominent in patients with large repeats, like congenital patients [29] and one group reported an increase in $(CTG)_n$-repeats in cerebellar cortex of DM1 patients as compared to other tissues [96].

Since DMWD has a much more widespread expression than DMPK and – based on our findings presented here - may have an important developmental role, it remains a strong candidate for being involved in (some of) the neuropathological aspects of DM1. Until now there have been two reports of changed DMWD expression in muscle tissue of DM1 patients or fibroblast cell-cultures obtained from DM1 patients [5,48]. Assuming that $(CTG)_n$-repeat expansion also affects DMWD expression levels in brain via cis-acting chromatin effects spreading throughout the entire DM1-gene cluster [165] how then could we explain the neuropathological changes seen in DM1 patients with respect to our findings on DMWD localization and expression? If we can extrapolate our findings in mouse to the situation in humans, we must assume that DMWD is expressed throughout the human brain in neurons in their cell body and nucleus, their dendrites and synapses, and also in the cortex layer II-VI in the cerebrum. As this area is known to be involved in cognition, abnormal DMWD expression may affect cognitive performance in DM1 patients.
Furthermore, synapses in general were found to play a very important role in sleep, memory and mood swings [109]. During sleep, self-generated electrical potentials (brain waves) were found to consolidate and reinforce memory circuits. When these brains waves did not occur, dedicated synaptic efficacies would be lost in these circuits, which could explain the memory loss and mood changes in DM1 patients [109].
The unique temporal regulation and spatial distribution of DMWD in the postnatal developing mouse brain, with strong expression in synaptic areas, suggests that DMWD has a very specialized function in neuronal development.
To verify the role of DMWD as a candidate for involvement in the neuropathological features of DM1, it will be of utmost importance to have a precise clinical, neurophysiological and histological description of all alterations that occur in congenital patients and in adult-onset patients at later age.

# Chapter 3

## DMWD is expressed in Sertoli cells and maturing sperm cells

J. Westerlaken[#] , P. Groenen* , D. de Rooij [±], P. de Boer [§], J. Hoogerbrugge [@] and B. Wieringa[#]



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**Summary**

RNA and protein products of the DMWD gene, located upstream of the DMPK gene in the myotonic dystrophy (DM1) gene cluster, occur in many tissues but most prominently in testis and brain, tissues that are known to be involved in disease manifestation in DM1 patients. The level of DMWD gene products is altered in DM1 patients [5,48].

To contribute to a better understanding of the possible pathobiological role of the DMWD gene, we report here on the fate of DMWD RNA and protein in the developing testis and cells derived thereof from mouse. DMWD reactive protein in testis extracts appeared with apparent molecular weights of 78 and 37 kDa, whereas DMWD mRNA was extracted as a transcript with uniform size. During the postnatal development of the testis the DMWD mRNA expression did not change dramatically. The 78 kDa protein product was already present at P0 and remained expressed at an equally high level during postnatal development, whereas the smaller 37 kDa product appeared at postnatal day P4 and kept increasing until day P21. In the testis, DMWD antigenic protein was located in very specialized structures like the sex vesicles in pachytene spermatocytes, elongating spermatids, and Sertoli cells. Based on our findings we propose that cell-type and post-translational regulation is involved in DMWD expression and that the DMWD gene is a possible candidate to play a role in the testicular atrophy of male DM patients.

## Introduction

Myotonic dystrophy (DM1) is a disorder that is caused by an unstable $(CTG)_n$-repeat located in the 3`-UTR of the Myotonic Dystrophy Protein Kinase gene (DMPK) [23,56,137]. Clinical manifestation in DM patients is highly variable and involves multiple systems and organs like muscle, brain, eyes, heart and testis [83]. Severity of the DM phenotype is correlated, but not absolute, with the repeat length in blood [25,82,93]. The pathobiological basis of the disease manifestation is still not very well understood, because some tissues affected in DM patients do not show any appreciable expression of the DMPK gene. For example, the expression level of $(CTG)_n$-repeat-containing DMPK transcripts is below detection level in testis, but 80% of male DM patients show a testicular atrophy.
Therefore, to study the possible involvement of other genes, several groups engaged in a study of neighbouring genes in the $(CTG)_n$-repeat area on chromosome 19. We concentrated on the myotonic dystrophy WD-repeat gene DMWD located just upstream of the DMPK gene. DMWD gene products are ubiquitously expressed with the highest product levels found in testis and brain. The DMWD protein contains four WD-repeats, units that are involved in protein-protein interactions, which makes this protein part of the WD-repeat protein family [158,199]. WD-repeat proteins form a propeller-like structure in which each WD-repeat represents one propeller blade. There is no obvious functional relation between WD-repeat protein family members and their biological function, although they can be classified into distinct sub-families, based on the proteins they interact with or on properties of other domains found in combination with the WD-repeats. Domains found together with the WD-repeats are the F-Box, the HMG-box and the SOCS box [10,28,88,229].
Here we report on the study of mRNA and protein products of the DMWD gene in testis and their possible role in disease manifestation in this tissue in DM patients.
DM patients undergo an apparently normal puberty, but then, during adulthood, testicular atrophy occurs along with a relative hypertrophy of Leydig cells. This process makes the testis small and soft, with tubular degeneration and hypospermatogenesis [83] as the most common features. In normal healthy men, as in all mammals, the process of cell division and differentiation in testis, called spermatogenesis, starts with undifferentiated spermatogonia that progress in mice over several weeks into spermatozoa (Fig. 1). Undifferentiated spermatogonia are located at the periphery of the testis tubules; during differentiation the germ cells move towards the centre of the tubule until spermatozoa are ultimately released into the lumen of the tubule. Undifferentiated spermatogonia undergo mitoses and part of them transforms via a variable number of cell divisions into differentiated spermatogonia type $A_1$, the remaining sub-population is self-renewing. Spermatogonia type $A_1$ give rise to $A_2$ – $A_4$, intermediate and type B spermatogonia. These spermatogonia type B divide to form meiotic cells, the primary spermatocytes, which progress during the first meiotic division into secondary spermatocytes. These secondary spermatocytes divide in the second meiotic division into round spermatids, which do not divide further but differentiate into elongating spermatids and ultimately into spermatozoa. In mice it takes approximately 3 cycles (approx. 26 days) of the seminiferous epithelium to develop from stem cell spermatogonium to round spermatid.
Spermatogenesis is a highly coordinated process. Every few days a new set of spermatogonia starts off to complete the spermatogenic cycle, i.e. one complete round of division and differentiation. Based on changes in acrosome and nucleus, the development of spermatids is divided into 16 steps. Steps 1 to 12 takes one spermatogenic cycle to finish and these steps are used to divide the spermatogenic cycle in 12 stages: I – XII. Cells represented in steps 1 to 8 are called round spermatids, cells in step 9 to 12 are named elongating spermatids and the cells in step 13 to 16 are the elongated spermatids.

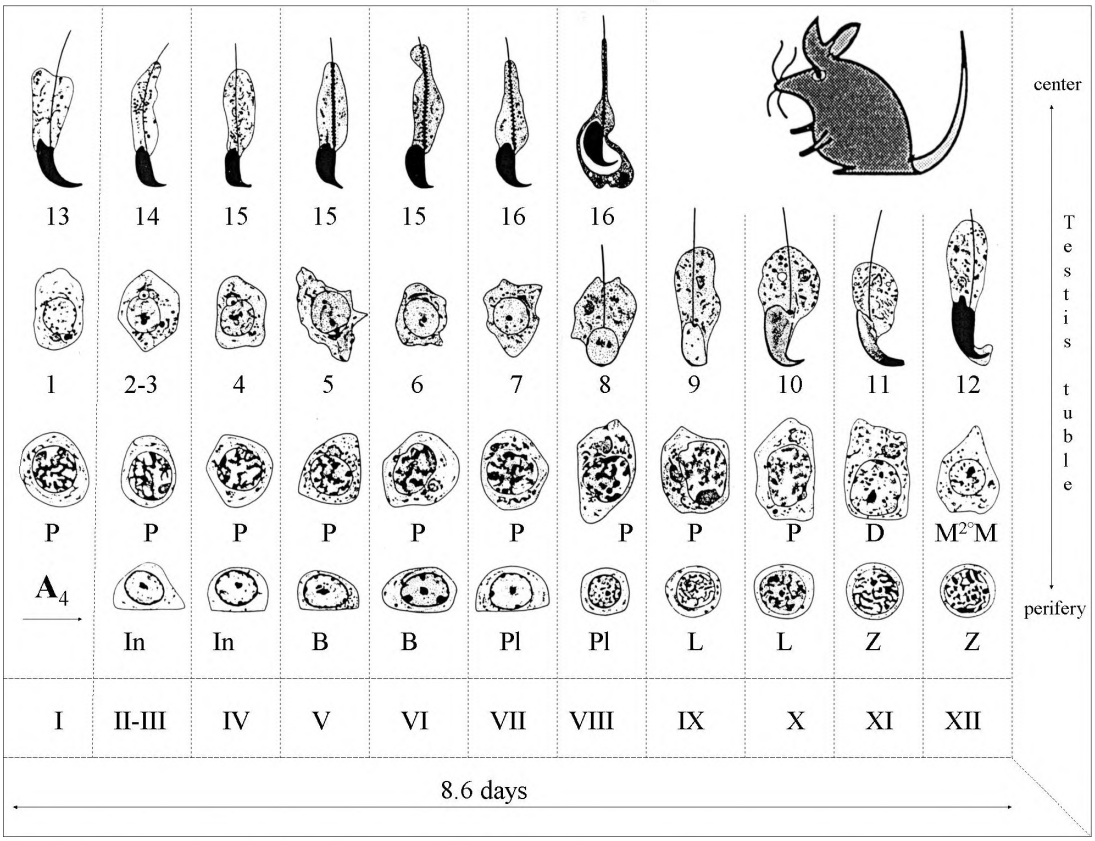


**Fig. 1.** Schematic overview of the spermatogenic cycle.
The 12 stages of the cycle are indicated with roman numerals. At the bottom of the figure it is shown that it takes 8.6 days for one spermatogenic cycle to finish. To the right is indicated were in the testis tube the cells are located.
Undifferentiated spermatogonia undergo mitoses and part of them transforms into differentiated spermatogonia type $A_1$, the other part renew their own population. Spermatogonia type $A_1$ give rise to $A_2$ – $A_4$, intermediate and type B spermatogonia. These spermatogonia type B divide to form meiotic cells, the primary spermatocytes, which progress in the first meiotic division into secondary spermatocytes, these secondary spermatocytes in turn divide in the second meiotic division into round spermatids. These round spermatids do not divide any more but differentiate into elongating spermatids and ultimately into spermatozoa. Based on changes in acrosome and nucleus, the development of spermatids is divided into 16 steps. Steps 1 to 12 take one spermatogenic cycle to finish and these steps are used to divide the spermatogenic cycle in 12 stages: I – XII. Spermatogenesis in total, from spermatogonia type $A_1$ to spermatozoa, takes approximately 35 days. This means that in every cross section of a tubule in testis you will find four differentiated cell-types next to the undifferentiated spermatogonia.
$A_4$: type $A_4$ spermatogonia, In: Intermediate type of spermatogonia, B: Type B spermatogonia, Pl: preleptotene spermatocytes, L: leptotene spermatocytes, Z: zygotene spermatocytes, P: pachytene spermatocytes, D: diplotene spermatocytes, primary spermatocytes: Pl, L, Z, P and D, $M^{2°}M$: secondary spermatocytes finishing meiosis, 1-8: round spermatids, 9-12 elongating spermatids, 13-16: elongated spermatids. Adapted from [186].

The spermatocytes are divided according to their stadium in meiosis of which the pachytene stage takes up almost half the total time needed to finish the first meiotic division. During this pachytene stage all homologous chromosome pairs are synapsed. The X and the Y chromosome form a special structure, which is called the sex vesicle or the XY body [163,183]. The sex vesicle is characterized by differential condensation of chromatin and transcriptional inactivity (for review see [201,202]) and is located at the nuclear periphery of pachytene spermatocytes in close association with the nuclear envelope [4,121].

In mammals the XY body formation appears to be essential for pachytene spermatocytes to proceed through meiosis [86]). Several authors have speculated about the function of the sex vesicle. McKee and co-workers proposed that the condensation of the sex chromosomes would prevent deleterious recombination events in non-homologous regions of the X- and Y-chromosomes [146].
Another suggestion was that the XY body enables and maintains synapsis of the heteromorphic X-and Y- chromosomes [136]. It is expected that the mechanisms controlling the XY body-formation are spermatogenesis specific and specifically associated with sex chromosomes [198].
In this study we followed DMWD mRNA and protein expression through various stages of postnatal testis development. Moreover, cell fractionation and immuno-microscopical analysis was used to follow the sub cellular partitioning of the protein at various stages in the spermatogenic cycle. We demonstrate here that DMWD mRNA is homogeneous and expressed at a constant level, but DMWD protein appears as a heterogeneous mixture of 78 kDa and 37 kDa products, which are differentially regulated during postnatal maturation of testis. Immunoreactive DMWD protein was located within the nucleus in the very specialized sex vesicles, but also in the cytoplasm of Sertoli cells. Spermatocytes and elongating spermatids were also found to contain DMWD mRNA and protein. Based on these findings we propose the DMWD gene as a candidate for being involved in the testicular symptoms in DM patients.

## Materials and methods

*DMWD antibody generation (abB13*
AbB13: A partial DMWD construct (amino acid 114 - 650) was created by the use of PCR with primer pat1 5′-GGA GAA TTC GAG CTT TAT TTC TAC CCC-3′ (nucleotide position 343 to 361) and pat2 5′-AGC GAA TTC TTA TCA CAC CAC AGT GCC-3′ (nucleotide position 1935 to 1953) on mouse cDNA clone 9.1 [99] (accession numberZ38011-Z38013 and Z38015). This PCR product was digested with EcoRI and ligated in frame into the EcoRI site of the pAr (delta EcoRI)(59/60) expression vector [18,209]. Next, the pARDMWD vector was introduced into E.Coli BL21(DE3)pLysS (Stratagene, California, USA) and induced with 0.33 mM IPTG (isopropylthio-β-D-galactosidase). The integrity of the induced protein (~60kDa) was checked by analysing a small protein sample on a 10% w/v SDS-polyacrylamide gel and the remainder was run on a large 10% w/v SDS-polyacrylamide gel for preparative purification. Protein with the correct size was excised from the gel and the protein was retrieved by electro-elution in a BIOTRAP BT100 apparatus (Schleicher & Schuell) according to manufacturer's protocols. The protein concentration was determined by the Lowry procedure according to Peterson [169] and protein purity was checked again by analysis on a 10% w/v SDS-polyacrylamide gel. A rabbit was immunized once with 400 µg of the purified DMWD protein in a 1:1 v/v ratio with incomplete Freund's adjuvant and three times with 300µg purified DMWD protein, also in a 1:1 ratio.
Two months later, after reactivity of the serum was checked with a Western blot on which the DMWD protein was blotted the rabbit was bled and the serum collected and affinity- purified over a column in which the truncated DMWD protein was bound to a matrix (Affi-gel10, Bio-Rad) in 0.1 M MOPS buffer according to the manufacturer's protocols. Purified antibody (designated abB13) was kept at –20 and –80°C.

*Western blot analysis of protein*

Tissues were collected from mice between 0 to 28 days after birth (P0 – P28). Distinct cell fractions were isolated (see isolation section) from testis of adult mice (6 – 10 weeks). Tissues and cell fractions were stored at –20°C until the day of use. Then, they were thawed and homogenized on ice in an appropriate volume of buffer (10 mM Tris pH 8, protease inhibitor cocktail (Boehringer Mannheim) and PMSF). Protein concentrations were determined by the Lowry procedure [169] and samples with an equivalent of 45 µg of total protein were analyzed by electrophoresis on a 10% w/v SDS-polyacrylamide gel and Western blotting. Therefore, proteins were transferred onto nitrocellulose membranes (Schleicher & Schuell, Keene, NH). Membranes were blocked in 5% skimmed milk in Phosphate Buffered Saline (PBS) with 0.05% Tween-20 (PBST) for minimal an hour at RT. Antibodies were diluted in fresh blocking solution and incubated o/n at 4°C on a rotating platform (abB13 1:5000). The blots were washed with PBST and incubated with peroxidase-conjugated goat anti-rabbit immunoglobins (Pierce, Rockford, IL) for minimal an hour at RT on a rotating platform, washed again with PBST and PBS and developed using a chemiluminescence Western blotting reagent.

Blocking experiments were done as follows. Normal procedures were followed up to the first antibody incubations. The abB13 antibody was incubated with either DMWD infected Sf9 cell-lysates or mock infected Sf9 cell-lysates (kindly provided by Dr. H. Hermsen), for one hour on ice and mixed every 5 min. The antibody mixture was centrifuged and the supernatant was used for incubation, which was done according the normal procedures (see above).

*Analysis of RNA from testis tissue and enriched cell fractions*

Total testis tissue was collected from a series of mice at different ages, between 1 to 28 days. Different spermatogenic cell types were obtained as enriched fractions from testis (see isolation section) from adult mice (6 – 10 weeks). Tissues and fractionated cells were snap-frozen in liquid nitrogen, thawed and homogenized on ice in an appropriate volume RNAzol™B (Campro Scientific) and further treated according to the manufacturer's protocol. Finally the RNA was dissolved in NSE (50 Mm NaAc, 0.2% SDS, 2 mM EDTA) and kept under 70% ethanol in NSE at –20°C until further use. RNA concentrations were determined by $E_{260\ nm}$/ $E_{280\ nm}$ readings and RNA integrity was checked on a 1% agarose test gel. For Northern analysis 10 µg of total RNA of each sample was analyzed by electrophoresis o/n on a 1% agarose/formaldehyde/ MOPS gel. After this a picture of the gel was taken and the RNA was transferred to a nitrocellulose membrane (Schleicher and Schuell), cross-linked by UV radiation, hybridized o/n at 65°C in a rotating chamber with a random labelled ($\alpha$-$^{32}$P-CTP, Amersham) 2.2 kb. EcoRI fragment containing DMWDfl as a probe. Bands on autoradiographs were visualized by exposure to Kodak X-OMAT film (18 to 72 hours).

*Isolation of spermatocytes, round spermatids and elongating spermatids*

Isolation of the different spermatogenic cells was done according to Grootegoed and Den Boer [74]. Briefly: Testis of C57Bl/6 mice were isolated and dissociated with different enzyme steps (collagenase, trypsin and hyaluronidase) and the use of physical forces (shaking and pipeting up and down in 10 ml pipets).

Pachytene spermatocytes, round spermatids and elongating spermatids were purified using sedimentation at unit gravity (Staput procedure), followed by density gradient centrifugation (Percoll gradients).

The different cell types were analyzed under a microscope and designated. The cell types used for RNA analysis were frozen at –80°C until further use. RNA was isolated with RNAzol (see RNA isolation section). The cell types used for protein analysis were frozen at –20°C until further use (see Western blot analysis of protein section).

*RT-PCR analysis of DMWD mRNA splice products*

One µg of DNAse treated RNA isolated from adult brain or testis was used as template to perform the RT-PCR reactions. Therefore, 100 ng of random hexamers was added to the RNA (total volume of 11 µl) and incubated at 65°C for 10 min and kept on ice afterwards. Four µl of 5xRT buffer (250 mM Tris pH8.3, 375 mM KCl, 15 mM $MgCl_2$), 2 µl 0.1M DTT, 1 µl 12.5 mM dNTP and 1µl mQ was mixed with the RNA mixture and incubated at 37°C for two min. One µl of LV-RT (200U) was added and the RT reaction took place for 1 hour at 37°C followed by 10 min at 65°C. Two µl of this RT reaction mixture was then used in a standard PCR-reaction with either primer combination A: JW8 5'-AGTGGCTGCCTGAGTCAGA GAG-'3 (nucleotide position 604 – 626; beginning of exon 3) and JW6 5'-TGCCTGGTTGGGAGGAGATG-'3 (nucleotide position 1907 – 1926; beginning exon 5) or B: JW5 5'-GCGAGGGCTTCTACAAGCTG-'3 (nucleotide position 40 – 60; beginning exon 1) and JW7 5'-CAGGCGTTCCTGAGCAATC-'3 (nucleotide position 1727 – 1746; end of exon 3) or C: JW5 5'-GCGAGGGCTTCTACAAGCTG-'3 (nucleotide position 40 – 60; beginning exon 1) and JW6 5'-TGCCTGGTTGGGAGGAGATG-'3 (nucleotide position 1907 – 1926; beginning exon 5). PCR products were run on a 1.5% agarose gel, transferred to a membrane (Schleicher and Schuell), cross-linked by UV radiation and hybridized o/n at 60°C in a rotating chamber with a random labelled ($\alpha$-$^{32}$P-CTP, Amersham) 2.2 kb EcoRI fragment containing DMWDfl. The signals were visualized by exposure to Kodak X-OMAT film (4 to 10 hours).

*Iimmunohistochemical analysis*

Mouse testes were fixed o/n in 10% buffered formalin and incubated in 70% alcohol for 2 hours, 30 min in 80% alcohol, two times 30 min and one time 45 min in 96% alcohol, two times 30 min and one time 45 min in 100% alcohol, two times in xylene for 35 min all at RT, 30 min in parafin (58°C), 2 hours in parafin (58°C) and embedded in parafin.

Tissue was cut in 5 µM slices, captured on slides and kept at room temperature until further use. On day of use slides were treated with xylol (3 times 5 min. each) and a graded ethanol series (5 min each; 100% to 50% ethanol). Slides were washed twice for 10 min in PBS (pH 7.4) and incubated in 0.35% $H_2O_2$ in PBS for 10 min at RT. Again the slides were washed again with PBS (3 times 5 min), permeabilized with 0.2% Tween-20 in PBS (PBST) for 10 min and blocked by incubation for an hour with 5% Normal Goat Serum (NGS) + 5% BSA in PBS. Then slides were incubated with the antibody abB13 (diluted 1:2 in 1% BSA in PBS), overnight in a humid chamber at RT. The next day the slides were washed in PBS (3 times 15 min.), incubated with goat-anti-rabbit biotin labelled secondary antibody (1:100 in PBS) (Pierce, Rockford, IL) for an hour at RT, washed again with PBS (3 times 10 min.), incubated with the ABC solution (Vector, elite kit) for 1 hour at RT, washed once with PBS and twice with 0.5 M Tris (pH 7.6) + 2.5 M NaCl + 1% Tween-20 (TBT) 10 min. each.

Presence of antigenic protein was made visible with DAB (3,3'-diaminobenzidine) according to the manufacturer's protocols. Finally, tissue sections on slides were incubated with DAB for 25-30 min at RT, washed with water, stained with Heamaluin for 1-2 min. washed with water (5 min.) and dehydrated through a graded 50% -100% ethanol series (5 min each) and in xylol (3 times 5 min.) and entrapped in Pertex.

*Immuno staining of fresh testis cells*

Staining of freshly isolated testis cells was done according to Baart, et al [9]. Freshly isolated spermatogenic cells were captured in fibrinogen/thrombin cloths, fixed with PFA and frozen at –80°C. On day of incubation, slides were slowly defrosted and immediately washed in PBS with 0.05% Triton (PBSTr).

Slides were blocked with 5% milk powder, 5% normal goat serum in PBSTr (blocking buffer) for 30 min. and subsequently incubated o/n in a humid chamber with the first antibodies (DMWD abB13, 1:50 and Cor1 1:100 in blocking buffer). The next day slides were washed with PBSTr and incubated with the second antibodies (FITC conjugated goat anti rabbit and TRITC conjugated goat anti mouse) for one hour, washed again, incubated with DAPI, washed and mounted in Vectashield (Vector). Slides were analyzed using a MRC1000 confocal laser-scanning microscope (Bio-Rad).

## Results

*Postnatal DMWD expression*

In previous studies [98] we had demonstrated that DMWD is prominently expressed in testis but its normal biological significance or its possible pathobiological involvement with problems in male DM1 patients remained unknown. To obtain clues for aspects of DMWD function we decided to first focus on the expression level and expression distribution of DMWD gene products during postnatal development of testis. For this purpose we isolated testis RNA and protein from male mice at various ages between P0 (day of birth) and P28 (28 days after birth). Because of the small size of the testicles when the mice were just born, we were unable to obtain sufficient RNA from the earliest time points. For this reason we could not load equal amounts of RNA for all samples and used normalization to total RNA content for comparing expression levels. As shown in Fig. 2, DMWD RNA was already present at P0 and expression levels stayed at approximately the same level throughout the first four weeks of postnatal development.

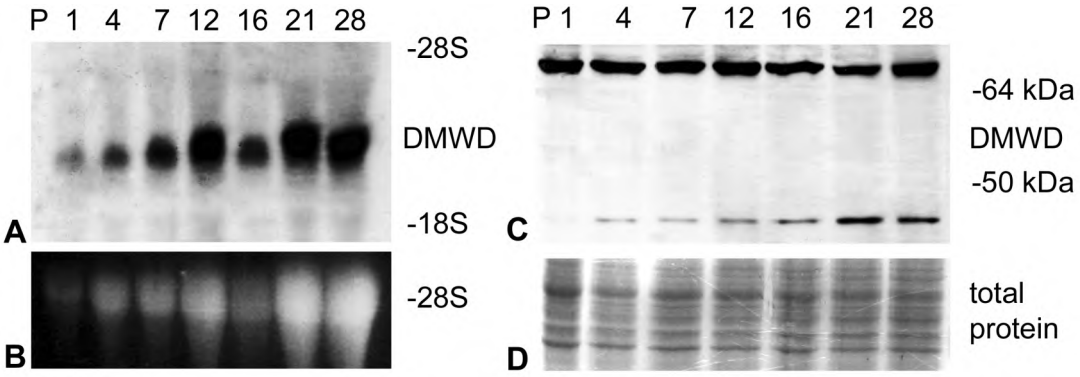


**Fig. 2.** Postnatal expression of DMWD
Panel A: DMWD RNA expression during postnatal development of the testis. Panel B: 28S RNA as a control for loading of total RNA. Panel C: DMWD protein expression during postnatal development of the testis. Panel D: Coomassie staining of total proteins as a control for equal loading. P 1 to 28: days after birth.

We also analyzed the postnatal DMWD protein content of testis during the same period. DMWD, on a Western blot, mainly appeared as two protein product bands, one of approximately 78 kDa and one of approximately 37 kDa. Subsequent analysis of the postnatal developing testis revealed that the larger 78 kDa protein gave the only detectable band at P0. The 37 kDa protein appeared just above background at postnatal day P4 and its expression levels increased during the next 24 days of postnatal development, whereas the 78 kDa protein concentration remained nearly constant during this period. To ascertain that the 37kDa signal resulted from specific binding of the abB13 antibody we performed antigen-blocking experiments.

In these experiments the abB13 antibody was pre-incubated with either baculovirus-produced recombinant DMWD in Sf9 cell-lysates or with mock infected Sf9 cell-lysates before using the antibody-lysate mixture on Western blot. In the presence of recombinant DMWD the 78 kDa and the 37 kDa bands disappeared completely, while both proteins could still be detected when blots were incubated with Sf9 extract only. It is of note here that DMWD appeared as a doublet of approximately 70 kDa protein products in brain. Again, the signal of these products disappeared in presence of DMWD protein in Sf9 cell-extracts. From these experiments we concluded that all proteins detected on Western blots with the abB13 antibody, the testis protein products of 37 kDa and 78 kDa and the brain 70 kDa doublet, were genuine DMWD-related protein products. The predicted size of the DMWD protein based on cDNA coding capacity and amino acid sequence composition was 69 kDa, which was close to that of the brain protein products but not the testis products, on Western blot. As we saw only one distinct DMWD mRNA band on Northern blots in both tissues, the most logical explanation for this observation was that DMWD is differentially processed and is undergoing phosphorylation (brain) or proteolytic cleavage (testis) or another type of post-translational modification.

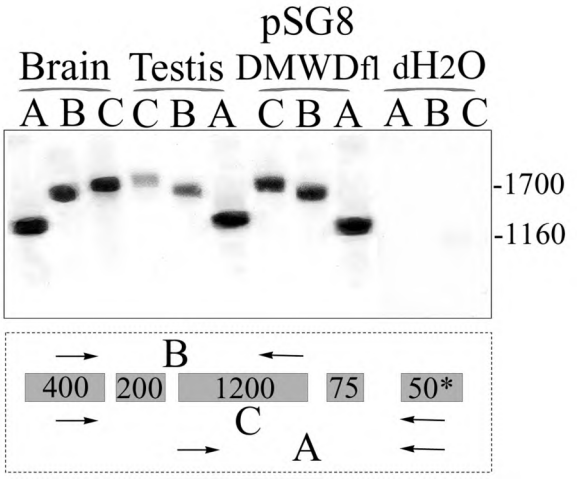


**Fig. 3.** RT-PCR products of DMWD in brain, testis, cDNA and control (water).
Alternative splicing was checked with three different primer combinations A, B and C, as schematically shown at the bottom of the figure, C giving the biggest product and A the smallest. The grey areas represent the exons.

*Alternative splicing*

Another explanation would be the occurrence of different open reading frames in mRNAs of nearly similar size. To determine if the different DMWD antigenic proteins could be the products of alternative splicing, we performed a RT-PCR assay across different exon combinations on RNA isolated from brain and testis (Fig. 3). In none of the tissues we could detect alternative splicing as PCR products from all regions were similar between brain and testis and all had sizes that were exactly identical to sizes of PCR products from DMWD cDNA (in pSG8 vector). This strongly suggested that the 37kDa testis DMWD protein was not a product of alternative splicing.

*DMWD expression in specific testis cells*

From P4 onwards the 37kDa DMWD protein was expressed at increasing levels during growth. To test the hypothesis that this small reactive product might be exclusively expressed in cell-types that develop, differentiate, or mature, during this period we fractionated early and late spermatocytes, round spermatids and elongating spermatids and analyzed the DMWD gene products in enriched preparations of these cell-types. As shown in Fig. 4 the DMWD RNA was present at comparable levels in all three cell-types.

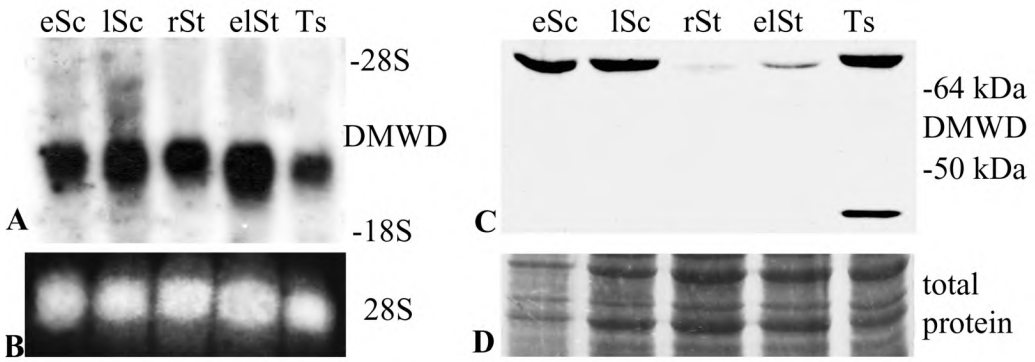


**Fig. 4.** DMWD expression in testis cell types.
Panel A: DMWD RNA expression in the different testis cell types. 10 mg total RNA was loaded in each lane. Panel B: ethidium stained 28S RNA is shown as control for equal loading. Panel C: DMWD protein expression in spermatocytes and spermatids. Panel D: Coomassie staining of total proteins is shown as control for equal loading. eSc: early spermatocytes, lSc: late spermatocytes, rSt: round spermatids, elSt: elongating spermatids, Ts: total testis lysates, mix: unfractionated mix of testis cells.

The small and large DMWD proteins however, displayed conspicuous differences in expression levels. The 78 kDa DMWD protein was mainly expressed in the spermatocytes (early and late; eSc and lSc), hardly expressed in the round spermatids (rSt) and found at a low level in the elongating spermatids (eSt). The smaller 37 kDa DMWD protein was not present in any of these cell-types. However, in an unfractionated mixture of testis cells we did detect the 37 kDa DMWD protein, suggesting that the 37 kDa DMWD protein resided in an as yet unknown cell type. From this experiment it was also clear that expression of the 78 kDa DMWD protein was not equally distributed over all spermatogenetic cell-types.

*DMWD immuno stainings*

To determine the cell type and intracellular distribution of DMWD we performed immunohistological stainings on adult testis tissue. This revealed that DMWD was mainly located in Sertoli cells and in the sex vesicles of pachytene spermatocytes (Fig. 5). Next to the Sertoli cells and the sex vesicles we also noted staining at times in type A spermatogonia (not shown) but as this staining was not consistent within a slide we scored them as negative for DMWD. In Sertoli cells the immunoreactive staining was found throughout the elaborated cytoplasm, which surrounds several spermatocytes and spermatids. The sex vesicle in spermatocytes is a structure in the nucleus located at the nuclear periphery that is formed by the X and the Y chromosome in the pachytene stage of meiotic prophase (reviewed in [201]). It can be easily distinguished from the autosomal bivalents [121]. DMWD was distributed uniformly throughout the whole sex vesicle but not detectably connected to any specific structure in the vesicle. The same sex vesicles were also clearly positive when we immunostained freshly isolated spermatocytes cells caught in fibrinogen/thrombin cloths with the abB13 DMWD antibody (Fig. 6). In these experiments we included the COR1 antibody, which recognizes the synaptonemal complexes (SC) of chromosomes in pachytene cells [45]. Cells that were stained with COR1 were also found positive for the DMWD proteins in the sex vesicles. Also in these experiments the DMWD staining was found in a smooth pattern throughout the entire sex vesicle. In (round or elongating) spermatids we could not detect any reliable staining for the DMWD protein. However, in the Western blot experiments described earlier (Fig. 4) we did detect DMWD protein in these cells.

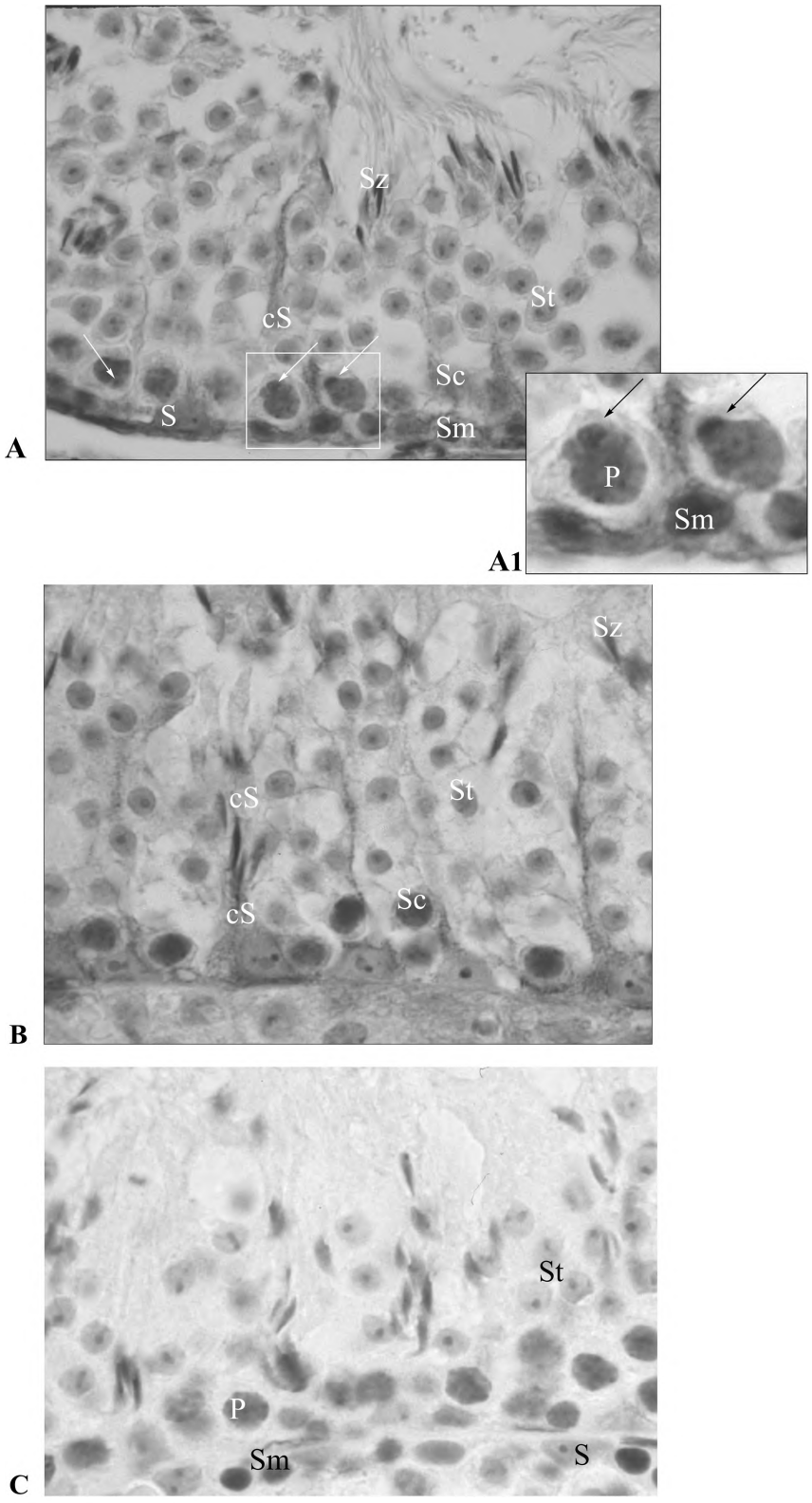


**Fig. 5.** Immuno stainings on testis tissue.
Panel A: staining of sex vesicles (arrows) and Sertoli cells (S). Panel $A_1$: enlargement of sex vesicles. Panel B: staining of Sertoli cells (S). Cytoplasm of these cells is indicated (cS). Panel C: Control slide stained as in A and B with omission of the DMWD antibody. Arrow: sex vesicle, S: Sertoli cells, cS: cytoplasm of Sertoli cell, Sm: spermatogonia, Sc: Spermatocytes, St: Spermatids, Sz: spermatozoa, P: pachytene spermatocytes.

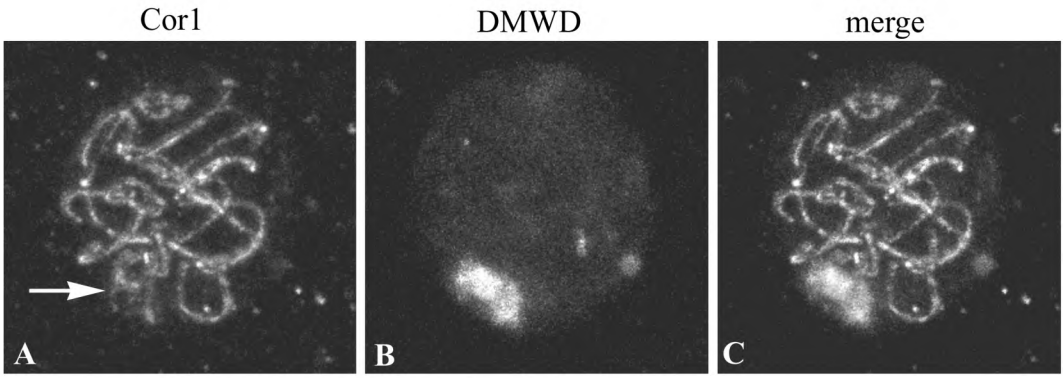


**Fig. 6.** Immuno staining of Pachytene Spermatocytes.
Panel A: Staining of pachytene spermatocyte with the Cor1 anti body. This anti-body recognized the synaptonemal complexes (SC) of chromosomes in pachytene cells [45]. Panel B: Staining of the same pachytene spermatocyte with the abB13 DMWD anti body. This anti body recognized the sex vesicle, a structure surrounding the X- and the Y-chromosomes in the pachytene stage of meiotic prophase. Panel C: Merge of the two anti body stainings, showing that the sex vesicle is surrounding the X and Y axial elements. The axial elements of the sex chromosomes are less intensively stained with the Cor1 anti body because their SC is not as extended as the autosomes. The arrow points at the X- and the Y-chromosome.

## Discussion

By revealing where in testis the DMWD RNA and proteins were expressed we hoped to reveal clues about DMWD function in this tissue. A major difficulty encountered in our study was the observation of gene product heterogeneity. DMWD immunoreactive proteins appeared as two distinct polypeptides, with molecular sizes of 78 and 37 kDa on Western blot, whereas DMWD mRNA appeared in one uniform size. The situation became even more complex when we drew a comparison to the DMWD-reactive proteins from brain extract, a 70 kDa doublet set of proteins. Because signals from all proteins could be blocked by pre-incubation with baculoviral-produced DMWD protein in Sf9 cell-lysates we assume that all products seen are indeed genuine products from the DMWD mRNA. Northern blot experiments and RT-PCR testing did not reveal heterogeneity at the mRNA level, strongly suggesting that the DMWD proteins are produced via post-translational modification. As the quantity of the 78 kDa protein remained at an equally high level during postnatal development, when the 37 kDa expression actually increased (Fig. 2), it was unlikely that there would be a precursor-product relationship between the 78 and 37 kDa proteins. Based on the cell fractionation studies shown in Fig.4, another possibility that is more likely is that 78 and 37 kDa proteins are products from different cell types.
In testis, the formation of different cell types is a highly delicate process (details in Fig.1) and therefore difficult to study. The development of spermatids is divided into 16 steps, steps 1 to 12 are used to divide the spermatogenic cycle in 12 stages: I – XII. The spermatids are divided into round spermatids (1 – 8) and elongating/elongated spermatids (9 – 16). The spermatocytes are divided according to their stadium in meiosis of which the pachytene stage takes up almost half the total time needed to finish the first meiotic division. During this pachytene stage the sex vesicle is formed [186]. We found that the 37 kDa DMWD protein was not expressed in any of the purified spermatocytes or spermatids (up to the elongating spermatids), ruling out these more developed spermatogenic cell stages as a source of this product. In a lysate of non-fractionated cells, also containing Sertoli cells, we did however observe the 37 kDa protein.

Moreover, when immunostaining on adult testis was performed we found DMWD immuno-reactive protein, which could be either the 78 kDa and/or the 37 kDa protein, mainly in the sex vesicles and in Sertoli cells. Combined, our findings suggest that Sertoli cells are the most important source of the 37 kDa protein and that the 78 kDa protein is mainly expressed in spermatocytes.
Another feature adding to this picture was the observation that the 37 kDa protein was increasingly expressed after postnatal day 4. It is known that Sertoli cells undergo a morphological and functional maturation process, which is dependent on ongoing spermatogenesis [73]. Although our results give only a descriptive picture for the expression of the 37 kDa protein, it is tempting to speculate about it having a specific role in the cytoplasm of Sertoli cells. Sertoli cells are the nursing cells of the testis in which the spermatogenic cells are embedded [73]. They have a very elaborate cytoplasm, which can support four round spermatids. Sertoli cell numbers determine the maximum number of germ cells per testis, which implicates the testis size and sperm output and creates the blood-testis barrier or Sertoli-cell barrier, by the formation of tight junctions between neighbouring cells. These junctions are established during maturation around the period when spermatogenesis starts and the first spermatocytes develop into spermatids. Germ cells cannot mature without Sertoli cells as they need the Sertoli cells for the production of lactate, amino acids and other materials [73]. Since Sertoli cells can have several specialized roles, the specific function of the 37kDa DMWD protein within these cells is not clear.
The 78 kDa DMWD protein was expressed from P0 onwards but this protein was mainly detected in the early and late spermatocytes and just a little in elongating spermatids (further differentiated than the round spermatids). The biggest population of spermatocytes in a single sub-stage is at the pachytene stage (see Fig. 5), which is the stage when the chromosomes are synapsed, showing a synaptonemal complex and the XY body/sex vesicle. The staining on freshly isolated pachytene spermatocytes also revealed the sex vesicle as DMWD positive (Fig. 5). There are several proteins known to localize to the sex vesicle like p51 [198] and XY77, which are homogeneously distributed throughout the sex vesicle. XY40 [4], another protein known to be located in sex vesicles, is selectively associated with the axial structures of the X and the Y chromosome. But the exact composition of the sex vesicle is not known and also appears to change during the life span of the sex vesicle [121]. The sex vesicle is a very specialized structure within the nucleus and is only present during the pachytene stage, this implies that DMWD, most likely the 78 kDa protein, has a very specialized role in meiosis during the synapsed stage of the sex chromosomes.

Although the distribution of mRNA does not always equal the distribution of protein, we examined the mRNA distribution as well. The DMWD RNA is equally expressed in all four cell-types as seen in Fig.4. Jansen and co-workers already showed, with RNA in situ hybridizations, that DMWD in testis is expressed in particular cell-types/testis developmental stages [98]. However, which types/stages exactly remains subject of discussion, as the interpretation of results shown in the Jansen 1995 publication, are not shared by others (Grootegoed and de Boer, personal communications). The uneven mRNA distribution is not seen on the Northern blots, which were made with the different spermatogenic cell types. However, it is possible that DMWD RNA is expressed more in certain sub-fractions of the isolated spermatogenic cell types, as the isolated cell types used here comprise 8 to 12 different developmental stages. This could give the even distribution seen on the Northern blots and an uneven distribution in the situ hybridization experiments.

*Immuno stainings*

The immuno-stainings on the testis sections and on the cloths revealed that DMWD was mainly expressed in Sertoli cells and in sex vesicles (Fig. 5 and 6). Other cell types, shown positive on the Western blots (Fig. 2 and 4), could not be detected with this method. We had noticed before, during other experiments, that the abB13 antibody was very sensitive for particular chemicals often used in biochemical and immuno histochemical procedures. In the immuno histochemical experiments we only seemed to detect some of the DMWD proteins, most likely the ones more highly concentrated, as we detected DMWD protein in early and late spermatocytes on Western blot but not on testis slides. It should also be noted that the Western blot procedure is more sensitive than the immuno procedure.

*DMWD and possible partner proteins*

One way to identify any partner proteins that engage in an interaction with DMWD is to investigate what is known about homologous proteins. Recently it became clear that the *A. nidulans* homologue of DMWD: CreC interacts with and stabilizes the deubiquinating enzyme CreB [134,217]. The CreB enzyme removes the ubiquitin moieties of CreA, a DNA-binding protein resulting in repression of the gene(s) under its control [134]. The human homologue of CreB is the human deubiquitinating enzyme UBH1 [80,133] and if we extrapolate the findings to the human situation, UBH1 could be (one of) the interactor of DMWD in testis and other tissues. If this interaction has the same nature as in *A. nidulans*, this would place DMWD in a regulating pathway. Another feature adding to this picture of an ubiquitin regulatory pathway is that genes in *C. elegans* operating in the same pathway are usually clustered together on the genome [105]. In *C. elegans* the homologue of DMWD: C08B06.7 is located next to an ubiquitin activating enzyme: aos-1, which could mean that they act in the same pathway.

Another possible interactor was found when a yeast two hybrid screening was done with DMWD (see chapter 4, this thesis). Human heterochromatin protein 1 gamma ($HPI^{hs}\gamma$) was found as a possible binding partner for DMWD. $HPI^{hs}\gamma$ is part of a heterochromatin sub-family which consists of three HPI proteins: $HPI^{hs}\alpha$, $HPI^{hs}\beta$ and $HPI^{hs}\gamma$. The three $HPI^{hs}$ variants are very similar in sequence and in mice 3T3 interphase cells, mHPIα and mHPIβ are found to localize at the exact same place within the nucleus [152]. M31, the mouse homologue of $HPI^{hs}\beta$ and $HPI^{hs}\beta$ and $HPI^{hs}\gamma$ but not the $HPI^{hs}\alpha$, were shown to be concentrated in the sex vesicle during spermatogenesis [150,155]. As shown in this chapter, DMWD protein is also located in this sex vesicle during spermatogenesis. Although their has been no report that $HPI^{hs}\gamma$ is also concentrated in the sex vesicle it is possible that at this particular moment in the cell cycle both $HPI^{hs}\beta$ and $HPI^{hs}\gamma$ are located in the same place.

Both possible DMWD interactor proteins, HPI and UBH1, are involved in gene regulation, which makes it likely that DMWD is also involved in gene regulation, either in a ubiquinitating pathway (CreC and aos-1) or by direct interaction with the DNA binding protein (HPI).

As stated before DMWD is located close to the expanded repeat in the DMPK gene and is highly expressed in testis, DMPK on the other hand is not expressed in testis. Therefore, we explored the possibilities of DMWD involvement in the testis-related symptoms seen in DM1 patients, based on the results shown in this chapter.

*Myotonic Dystrophy and DMWD expression*

One hypothesis of the disease manifestation of DM1 is that the expanded CTG repeat in the DMPK gene might alter expression of not only the DMPK gene, but also neighbouring genes like SIX5 and DMWD, due to epigenetic effects [165].

Another possibility is that the DMWD and DMPK transcripts become "contiguous" when transcription of DMWD is not terminated properly, as reported (although under artificial circumstances) by Groenen and co-workers [71]. These two hypotheses leave interesting possibilities for involvement of DMWD in the DM manifestation.
Eighty percent of the male DM patients show a testicular atrophy even though they undergo an apparent normal puberty, testicular atrophy occurs along with a relative hypertrophy of Leydig cells. The testes are small and soft with tubular degeneration and hypospermatogenesis [83]. As shown in this chapter, DMWD proteins are expressed in Sertoli cells, in the sex vesicle (spermatocytes) and in the elongating spermatids. Sertoli cells are the nursing cells of the testis and provide materials to the developing spermatocytes; the germ cells cannot mature without them. The Sertoli cell number determines the maximum number of germ cells per testis, which implicates the testis size and sperm output [73]. If DMWD expression in Sertoli cells would be changed, as a result of the expanded CTG repeat in DM patients, Sertoli cells might not be able to nurse the developing spermatocytes anymore which could account for the degeneration and hypospermatogenesis seen in these patients. DMWD protein(s) located in the sex vesicle might also play a role in this, as in mammals the XY body formation appears to be essential for pachytene spermatocytes to proceed through meiosis [86]). If the sex vesicle is not formed, the sperm will also not mature and degenerate.
There have been two reports of changed DMWD expression in DM patients or cell-cultures obtained from DM patients. Alwazzan, 1999 reported that the level of DMWD RNA from the expanded repeat allele in the cytoplasmic fraction of DM cell lines is reduced compared to the level of DMWD RNA from the wild type allele, implicating that expression of DMWD changes in DM patients. Eriksson, 1999 also reported a decreased expression of the DMWD RNA in DM patients, compared to non-DM patients. Although we do not know if the DMWD expression of DM patients in testis is changed, it might very well be possible that DMWD is involved in the manifestation of DM with respect to the testis, based on the sub testis cell-type and intra-cellular localization of the DMWD proteins.

Based on the results shown in this chapter we would like to propose that the 37 kDa DMWD protein is most likely expressed in the (more matured) Sertoli cells were it will have a very specialized function. The larger DMWD protein is most likely expressed in the spermatocytes and elongating spermatids were it is possibly involved in meiosis and development of the spermatids. Based on the localization of the DMWD proteins in testis, the two reports on changed DMWD expression in DM patients, and the absence of DMPK expression in testis, we would like to propose that DMWD is a very good candidate gene to be involved in the testicular atrophy seen in DM patients.

# Chapter 4

## The heterochromatin protein family member $HPI^{Hs}\gamma$ is a binding partner for the DMWD protein encoded in the myotonic dystrophy gene cluster

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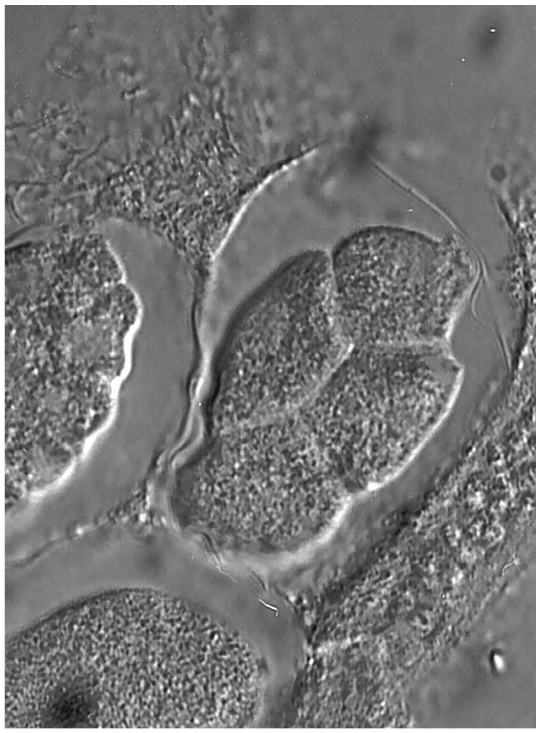


**Acknowledgements:** We thank P. Groenen and R. Wansink for their helpful advice. We thank H. Hermsen for his help with the production of native DMWD protein in the Sf9-baculovirus expression system

**Summary**

The DMWD gene is a member of the myotonic dystrophy (DM1) gene cluster on human chromosome 19q, located just upstream of the myotonic dystrophy protein kinase (DMPK) transcription unit. The gene is considered a candidate for being involved in the complex DM1 disease phenotype because its expression is altered in some DM1 patients [5,48]. Its chromosomal location and typical expression pattern, which is ubiquitous but most prominent in brain and testis, and also the unique structural features of the protein product make the DMWD gene an interesting target for further study.
Here we report on the characterization of the molecular environment of the DMWD protein as an approach to reveal clues about its cellular function. Sizing of the native DMWD protein in extracts of different tissues with the help of column chromatography revealed that DMWD is part of large macromolecular complexes, with MWs ranging between 270 kDa in brain to 15.000 kDa in testis. Use of the yeast-two-hybrid approach for the identification of interacting partner proteins revealed that the N-terminal part of DMWD is able to associate with members of the heterochromatin protein I family of proteins (HPIγ, HPIα). By tracing GFP-tagged protein segments with fluorescence microscopy in transfected COS-1 cells it was demonstrated that the truncated DMWD consisting of the N-terminal part specifically co-localized with HPIγ in the nucleoplasm. However, full length DMWD did not show appreciable binding to HPI family members and was located predominantly in the cytoplasm. Our results suggest that DMWD may be conditionally involved in coordinating HPI protein dynamics. We discuss our findings with respect to new knowledge on the role of the DMWD homologous protein CreC in *Aspergillus nidulans*.

## Introduction

Myotonic dystrophy (DM, also know as Steinert disease) is an autosomal dominant multi-systemic disorder and the most common form of muscular dystrophy in adults with an incidence of 1: 8000 [83]. The most common form of disease, DM-type 1 (DM1), is caused by an unstable $(CTG)_n$-repeat located in the 3 `UTR of the Myotonic Dystrophy Protein Kinase gene (DMPK) [24,56,137]. In 1998 a second DM locus was identified on chromosome 3q and recently it was published that this form of disease, DM-type 2 (DM2), is caused by a $(CCTG)_n$ expansion in intron 1 of the zinc finger protein 9 (ZNF9) [131,177]. Myotonic dystrophy affects many different tissues and organs, including muscle, brain, eyes, testis and heart [83]. Based on several studies there is now consensus that $(CTG)_n$-repeat expansion in DM1 may exert a dominant gain-of-function effect on the expression of multiple genes. Mechanistically, $(CTG)_n$-repeat size increase may (i) cause chromatin topology distortion, resulting in altered transcription of either one of three genes from the DM1 locus (the DMWD gene, the mutant DMPK gene itself, or the Six5 gene (formerly called DMAHP [21]) or (ii) lead to titration of pre-mRNA splicing or binding factors, possibly affecting the profile of alternative splice products of many genes [170,214,215].
The DMWD gene might be involved in the manifestation of DM because it is located just upstream of the DMPK gene and is expressed in tissues that are affected in DM1 patients -like brain and testis- tissues where the DMPK gene is not expressed. These facts plus the rather unique structural features of the DMWD protein make it interesting in its own right. The protein consists of a proline rich N-terminal part, four clearly distinct WD-repeat units and a C-terminal end with unspecified properties. WD-proteins usually contain 4 - 8 WD-repeats, which fold into twisted propeller like structures thought to be involved in protein-protein interactions. Although there is not a common function for the WD-repeat segment itself, the protein family can be divided in subfamilies based on the classes of proteins they interact with or the other domains they contain. One of the best-studied WD-repeat proteins is the Gβ subunit of heteromeric GTP binding proteins (G-proteins). This protein is built up of 7 WD-repeats and interacts with the pleckstrin homology (PH) domain of src-related tyrosine kinase TecIIa and the GTPase dynamin via the WD-repeats. Also other WD-proteins are known to bind to pleckstrin homology domains and may provide a regulatory link between signal transduction and cytoskeletal function [61,225,226]. A second class of WD-repeat proteins may link control and regulation of the basal transcription machinery by gene-specific transcription factors. The best example for this class is the WD-repeat/TRP interaction first identified in Tup-1[116,117,212]. The third class of WD-proteins may link signal transduction and gene expression by interacting with heterologous nuclear ribonucleoprotein K proteins [42]. Finally, other sub-families consist of the WD-repeat domain connected to distinct other domains like the F-Box, the HMG-box or SOCS box. WD-repeat proteins with an F-box (named Fbws [28]) mainly act as regulators like Cdc4p, that controls cell division by degrading G1 cyclins and cyclin dependent kinase inhibitors [41] or Met30p which is required for repression of methionine genes [212]. Proteins with an HMG-box (a 80 amino acid motif) bear a flattened triangular fold structure that recognizes and binds architectural features in DNA [115,229]. Thus, WD proteins with an HMG-box combine direct DNA binding with protein-protein binding properties. Proteins that contain a SOCS-box together with WD-motifs are called WSB proteins. The SOCS motif was first identified in the suppressor of cytokines signalling family [88]. Members of this family are known to inhibit cytokine or growth factor (GF) signalling via the JAK/STAT (Janus kinase/ signal transducers and activators of transcription) pathway [33].

As homology comparisons to any member of these WD protein subfamilies did not gave us any insight in functional properties we decided to study the molecular environment of DMWD. Identification of protein complexes and partner-binding proteins may reveal clues for biological significance and teach us more about DMWD's possible candidacy for the DM disease phenotype. We show here that DMWD in testis forms part of a very large protein complex with a molecular weight (MW) of approximately 15.000 kDa. In brain DMWD was located in a smaller complex with a MW of approximately 270 kDa. Protein complexes in these tissues were very stable as they were resistant against treatment with detergents or high salt concentrations. When we screened a human brain library for possible interacting proteins the HPIγ and HPIα members of the heterochromatin-associated protein family were identified as putative binding partners for the N-terminal part of DMWD. Fluorescence microscopic tracing of GFP-tagged segments of the DMWD protein revealed that this N-terminal HP1-binding domain co-localized with HP1-family members in the nucleoplasm whereas full length DMWD was located predominantly in the cytosol.

## Materials and methods

*Tissue extraction and gel-sizing of DMWD containing protein complexes*

Whole brain, testis and intestine of adult mice were isolated and immediately frozen in liquid nitrogen. Tissues were thawed on the day of use and homogenized with a mixer on ice in an appropriate volume (0.5-2 ml) of buffer (10 mM Tris pH 7.4, 0.15 M NaCl, containing a cocktail of protease inhibitors (Boehringer Mannheim, Indianapolis, USA). Next, samples were centrifuged at 13.000 rpm for 20 min. at 4°C and the supernatant collected. Protein concentrations were determined by the Lowry procedure according to Peterson [169], and samples stored at –20°C until further use. Samples were treated with detergents (NP40, Triton X 100, SDS) or high salt (all solved in 10 mM Tris pH 7.4, 0.15 M NaCl) by diluting the samples with a concentrated salt/detergent stock solution (usually 1:1) to get a final concentration of 8µg/µl total protein for brain and intestine and 5µg /µl for testis. Incubations were done on ice for one hour. Each sample was centrifuged again just prior to gelfiltration and 50µl was injected onto a Superose 6PC3.2/30 column (Pharmacia LKB Biotechnology, Uppsala, Sweden) while buffer (10 mM Tris pH 7.4, 0.15 M NaCl) was already running over the column. The flow was kept steady at 40µl/min and fractions of 65µl were collected in eppendorf tubes. After each run the column was washed with at least twice the bed volume before a new sample was applied. To control and calibrate column performance a mixture of marker proteins (Thyroglobulin dimer 1338 kDa, Thyroglobulin monomer 669 kDa, Ferritin 440 kDa, Immunoglobulin G 160kDa, BSA 67 kDa, Chymotrypsinogen A 25kDa and RibonucleaseA 13.7 kDa) was run on the column both prior to and after the elution session. The contents of the 65µl fractions were freeze dried and dissolved in sample buffer (100 mM Tris-HCl, pH 6.8, 200 mM dithiothreitol, 4% SDS, 0.2% bromophenol blue, 20% glycerol), 7.5 µl (for testis and intestine) or 15µl (for brain). Proteins in 7.5 µl of each sample were resolved on 10% PAA-SDS gel before transfer to nitrocellulose membrane by Western blotting.

*Protein analysis on Western blots*

For direct analysis of DMWD tissue distribution, tissues were collected from adult mice and stored at –20°C until the day of use. Then tissues were thawed and homogenized on ice in an appropriate volume of buffer (10 mM Tris pH 8, protease inhibitor cocktail (Boehringer Mannheim) and PMSF) and protein concentrations were determined [169].

Of each sample, 40 µg of total protein was analyzed by electrophoresis on a 10% w/v SDS-polyacrylamide gel and subsequently transferred onto nitrocellulose membranes (Schleicher and Schuell, Keene, NH).

*Immune-detection of DMWD protein on Western blots*
Membranes were blocked in 5% skimmed milk in Phosphate Buffered Saline (PBS) with 0.05% Tween-20 (PBST) for minimal an hour at RT. Antibodies were diluted in fresh blocking solution (anti-DMWD abB13 (1:5000), anti DMWD abH84 (1:5000) and anti-GFP (1:5000) and the mouse monoclonal P5D4 (1:10.000)) and incubated with the blot membrane o/n at 4°C on a rotating platform. The blots were washed with PBST and incubated with peroxidase-conjugated goat anti-rabbit immunoglobins or peroxidase-conjugated goat anti-mouse IgG (Pierce, Rockford, IL) for minimal an hour at RT on a rotating platform. Subsequently the blots were washed with PBST (twice) and PBS (once) and the labelled protein bands were visualized using freshly prepared chemiluminescent substrate (100 mM Tris-HCL, pH 8.5, 1.25 mM p-coumaric acid (Sigma), 0.2 mM luminol (Sigma), 0.009% $H_2O_2$) and exposed to Kodak X-omat autoradiography films.

*Screening of DMWD binding proteins with the Yeast two hybrid Interaction Trap*
The yeast strain and the cDNA library used for the interaction trap assay were provided by Dr. Roger Brent and colleagues (Massachusetts General Hospital, Boston) and used as described [76]. Briefly, three different cDNA fragments encoding the N-terminus, the WD-repeat segment and the entire ORF of DMWD were introduced in the bait vector pMW101 [228]. To generate the three different DMWD bait-plasmids the following cloning steps were used. The cDNA segment encoding the N-terminal domain of 104 amino acids (7 to 111), vector pMW101-$DMWD_{n7\text{-}111}$, was made by PCR with primers: 5′-CG-GAA-TTC-ATC-AAG-TCG-CAG-TTT-CG-3′ (complementary to nucleotide positions 19-35) and 5′- CG-CTC-GAG-TTA-CAG-GTT-GAA-GCA-GAC-C-3′ (complementary to nucleotide positions 340-359) on pSG8DMWDfl plasmid DNA as template (see *DMWD expression constructs* below). Next the PCR fragment and the pMW101 vector were digested with EcoRI and XhoI, and the fragment was ligated into the vector, using standard procedures. The cDNA segment encoding a WD-repeat spanning domain of DMWD of 261 amino acids (192 to 453), vector pMW101-$DMWD_{wd192\text{-}453}$, was generated by PCR with primers: 5′-CG-GAA-TTC-ATT-GAC-AAG-ACC-AAG-GTG-3′ (complementary to nt positions 576-594) and 5′-CG-GGA-TCC-TTA-AGA-ACT-ACC-AGA-AGC-TGG-3′ (complementary to nt positions 1347-1368). The PCR fragment was digested with restriction enzymes EcoRI and BamHI and the fragment was inserted in EcoRI/BamHI digested pMW101. To generate pMW101-$DMWD_{fl1\text{-}650}$, a bait vector with a full-length DMWD cDNA insert, plasmid pSG8DMWDfl was cleaved with EcoRI to yield a fragment spanning the complete DMWD ORF (amino acids 1 till 650). Next, this DMWDfl insert was cloned in the appropriate orientation into the EcoRI site of vector pMW101 vector. All plasmid DNAs were checked by restriction enzyme digestion and sequencing prior to use. These three bait-vectors were used to screen the human fetal brain library 4B in the prey vector pJG4-5 [76] for possible binding partners. Plasmids were introduced in yeast strain EGY48 (MATa trp1 ura3 his3 LEU2: :pLexAop6-LEU2) and tested for LEX A trans-activating interactions on minimal agar plates lacking tryptophan, histidine, uracil, and leucine but containing 2% galactose, 1% raffinose, and 80 µg/ml 5-bromo-4choloro-3-indolyl-beta-Dgalactopyranoside (X-gal). After the first round of screening, cDNAs of all positive clones were isolated and transformed back into yeast strain EGY48 with bait (pMW101) and reporter (pMW107) vectors [76,228].

The cDNAs of all positive clones were isolated and the 5'-sequences were determined with the 5'-BCO (5´-CCA-GCC-TCT-TGC-TGA-GTG-GAG-ATG-3´) primer and the Thermal Sequenase cycle-sequencing kit (Amersham Life Science) according to manufacturer's protocols. Sequences were determined on the semi-automated ALFexpress apparatus (Amersham Pharmacia Biotech, UK).
From $10^6$ transformants of the human fetal brain library, 16 clones were retrieved showing an interaction with the N-terminal part of DMWD, 3 clones were recovered showing an interaction with the WD-repeats of DMWD and no clone was found to interact with the full-length protein. Sequences were identified by using the BLAST program at the BCM search launcher (http://searchlauncher.bcm.tmc.edu/seq-search/) with the nr database (contains all GenBank+EMBL+DDBJ+PDB sequences). Sequences with high possibility scores were aligned with the proposed protein to confirm the hit (also done at the BCM site http://searchlauncher.bcm.tmc.edu/seq-search/alignment.html). Potential interesting positive prey cDNAs were then cloned between the EcoRI/XhoI sites of bait vector pMW101 and transformed into EGY48 together with prey plasmids containing the appropriate DMWD cDNA fragment and reporter vector to confirm the interaction. Only those cDNAs that showed an interaction in both the bait and prey vector environment, where further analyzed.

*DMWD expression constructs*
To study the intracellular localization behaviour of the different domains of DMWD by transfection into COS-1 cells we generated plasmids that were either based on the modified eukaryotic expression vector pSG8-VSV [36] or the modified eukaryotic vector pSG8△EcoRI. The pSG8△EcoRI vector is based on pSG5 [70], it has an extended multiple cloning site (inserted into the EcoRI/BglII, containing 6 unique restrictions sites, EcoRV, HinDIII, ClaI, SacI, XhoI and DraII). The vector for expression of the N-terminal DMWD domain was made by cloning nucleotides 1 to 404 into the pSG8-VSV vector in frame, which comprises DMWD from amino acid 1 to 135 (pSG8-VSV-DMWD$_{1\text{-}135}$). The pSG8-DMWD$_{fl}$ vector was obtained by linking two EcoRI sites to the complete ORF of DMWD, and cloning this fragment in frame into the pSG8ΔEcoRI vector (pSG8-DMWD$_{fl}$). The insert in this vector contains the full-length DMWD protein from amino acid 1 to 650.

*HPI constructs*
To check for co-localization of the prey protein, we generated three vector plasmids encoding EGFP-tagged versions of HPI$^{Hs}\gamma$. These vectors were all based on the eukaryotic expression plasmid pEGFP-C1 (Clontech, California, USA) and were made by insertion of an EcoRI/XhoI cDNA fragments from the prey vector (pJG4-5) [76] containing, either the full-length HPI$^{Hs}\gamma$ (no.2: pEGFP-HPI$_{fl}$), HPI$^{Hs}\gamma$ from amino 5 to end (no. 26: pEGFP-HPI$_{5\text{-}173}$) or HPI$^{Hs}\gamma$ from amino acid 77 to end (no. 21: pEGFP-HPI$_{77\text{-}173}$).

*Protein localization by transfection into COS-1 cells*
Transfection into COS1 cells to study the actual distribution of the different protein products was done by using either the electroporation or DEAE-dextran method [89]. All vectors used were isolated and purified by the Qiagen miniprep kit according to manufacturer's protocols. For each electroporation 5-10 µg pure circular plasmid DNA was used. Prior to use cells were grown in 10% FCS DMEM medium until 60-70% confluency, in 9 cm culture disks. On the day of transfection the cells were given fresh medium two-three hours before start of the experiment. Then cells were washed, treated with trypsin, washed again and counted, and for each transformation ± 1.5 $10^6$ cells in 200 µl PBS was used. DNA plus the cells were mixed in an electroporation cuvet on ice.

Electroporation was performed in a Bio-Rad GenePulser at 0.3V and 125µF. Thereafter cells were mixed with 10% FCS medium and plated on glass slides in 24 wells plates. After 24 hours of growth at 37°C, cells were fixed before staining.
For transfection with the DEAE-dextran method, cells were grown in 10% FCS DMEM medium until 60-70% confluency, in 9 cm culture disks. On the day of transfection the cells were given fresh medium two-three hours before the start of the experiment. Transfection started with washing the cells with serum free medium (Optimem), then the DNA/DEAE-dextran mixture (5-10 ug DNA, 9 µl cloroquine (2 mM in MQ), 300 µl DEAE-dextran (1 mg/ml) and 5.7 ml Optimem) was gently placed on top of the cells and let stand for two hours at 37°C. Subsequently the DNA/DEAE-dextran mixture was removed and the cells were shocked for 2 min. at room temperature with 10% DMSO in PBS. The DMSO solution was replaced with 10% FCS DMEM medium and the cells were grown at 37°C. After 2-3 hours the medium was replaced and the cells were further grown overnight at 37°C. At 20-24 hours after transfection the cells were fixed and stained.
Cells transfected only with pEGFP-$HPI_{fl}$, pEGFP-$HPI_{5-173}$ or pEGFP-$HPI_{77-173}$ were washed twice with PBS, fixed with 3% paraformaldehyde in PBS for max. 5 min, washed again with PBS and directly embedded in Mowiol (Sigma Chemical). Cells transfected with only pSG8-VSV-$DMWD_{1-135}$ or pSG8-$DMWD_{fl}$, or together with one of the HPI vectors (pEGFP-$HPI_{fl}$, pEGFP-$HPI_{5-173}$ or pEGFP-$HPI_{77-173}$) were washed twice with PBS, fixed with 3% paraformaldehyde in PBS for max. 5 min and washed again with PBS. Subsequently the cells were permeabilized with 0.5% NP-40 in PBS for 5 min, washed with PBS and incubated with 100mM glycine in PBS. Next cells were pre-incubated with 1% NGS in PBST for an hour and subsequently incubated overnight with the appropriate antibody (abH84 1:200, abB13 1:200, anti-VSV 1: 100 or anti-GFP 1: 200) in a humid chamber. The following day cells were washed with PBS and incubated with the secondary antibody (GAR-FITC or GAM-TXR, Jackson ImmunoResearch Laboratories, USA) for an hour in a humid chamber. They were washed again and embedded in Mowiol (Sigma Chemical). All cells were kept in a cool and dark place until being analyzed with a confocal laser-scanning microscope (MRC1000, Bio-Rad).

*Immuno pull down assay*
Cos-1 cells were cultured in DMEM/10% fetal calf serum and transfected by the DEAE-dextran procedure (described above) with one of the following vector combinations:

- pSG8-$DMWD_{fl}$
- pSG8-VSV-$DMWD_{1-135}$
- pEGFP-$HPI_{fl}$
- pEGFP-$HPI_{5-173}$
- pEGFP-$HPI_{77-173}$
- pSG8-$DMWD_{fl}$ and pEGFP
- pSG8-VSV and pEGFP-$HPI_{fl}$
- pSG8-$DMWD_{fl}$ and pEGFP-$HPI_{fl}$
- pSG8-$DMWD_{fl}$ and pEGFP-$HPI_{5-173}$
- pSG8-$DMWD_{fl}$ and pEGFP-$HPI_{77-173}$
- pSG8-VSV-$DMWD_{1-135}$ and pEGFP-$HPI_{fl}$
- pSG8-VSV-$DMWD_{1-135}$ and pEGFP-$HPI_{5-173}$
- pSG8-VSV-$DMWD_{1-135}$ and pEGFP-$HPI_{77-173}$
- pSG8-VSV-$DMWD_{1-135}$ and pEGFP

After transfection cells were plated on a 10-cm dish and cultured for 24 hours in DMEM/10% fetal calf serum. Cells were washed with cold PBS and lysed on plate with 550 µl ice-cold RIPA buffer (50 mM Tris, pH 8.0, 100mM NaCl, 1 mM EDTA, 1% NP-40, 0.1% SDS, 0.5% DOC, 1 mM PMFS and protease inhibitor cocktail (Boehringer)). After one-hour incubation on ice, the lysates were centrifuged for 10 min. at 10,000 g at 4°C and proteins in 50 µl of the lysates were separated for further analysis.

The remaining of the lysates were incubated on a rotating platform with 2 µl of abB13, 2 µl of abH84 (polyclonal anti-rabbit, both anti-DMWD described in chapter 2, this thesis), 2 µl of anti-VSV (monoclonal mouse P5D4 [122]) or 2 µl of anti-GFP (polyclonal anti-rabbit, [37]) at 4°C. After 4 hours, 50 µl of protein A-Sepharose CL-4B (Pharmacia) was added and incubation was prolonged overnight. The protein A-Sepharose bound immuno-complexes were washed four times with 1 ml RIPA lysis buffer and boiled for 5 min in 50 µl of sample buffer (100mM Tris-HCL, pH 6.8, 200 mM dithiothreitol, 4% SDS, 0.2% bromophenol blue, 20% glycerol). Proteins in fifteen µl of lysates and immunoprecipitates were resolved on 10 or 15% polyacrylamide gels and transferred to nitrocellulose membranes by Western blotting. Western blots were processed as described above with the following dilutions for antibodies: (i) rabbit polyclonal abB13 (1:5000), (ii) abH84 (1:5000), (iii) anti-GFP (1:5000) and (iv) mouse monoclonal P5D4 (1:10.000).

*Computer analysis*

Analyzes of sequences of DMWD protein segments were done as follows: The DMWD protein sequence was scanned with ScanProsite at httt://www.expasy.ch/cgi-bin/scanprosite (database:prosite release 17-april-2002) and with http://hits.isbsib.ch/cgi-bin/PFSCAN_parser (databases: prosite patterns (weekly-updated), prosite profiles (weekly update) and Pfam collection of hidden Markov models (weekly update) for possible phosphorylation, glycosylation and myristylation sites. The protein was also analyzed for any sorting signals with the program PSORT II at http://psort.nibb.ac.jp/cgi-bin/runpsort.pl. This site uses NUCDISC to discriminate for nuclear localization signals. PSORT uses the following two rules to detect an NLS: 4 residue pattern (called “pat4”) composed of 4 basic amino acids (K or R), or composed of three basic amino acids (K or R) and either H or P; the other (called 'pat7') is a pattern starting with P and followed within 3 residues by a basic segment containing 3 K/R residues out of 4. Another NLS pattern (called “bipartite”) is: 2 basic residues, 10-residue spacer, and another basic region consisting of at least 3 basic residues out of 5 residues. NES sequences were analyzed by computer analysis of hydrophobic amino acids.

## Results

*DMWD protein complexes*

Northern blot analysis on several mouse tissues revealed that the DMWD RNA was expressed at a high level in brain and testis and at a low level in many other tissues. Denaturing gel electrophoresis and Western blot analysis of DMWD in protein extracts revealed several protein products in brain and testis but also in other tissues with low RNA expression. The proteins in testis, tissues of the gastro-intestinal tract, bladder and uterus were represented by two bands of approximately 78 and 37 kDa (Fig. 1). In contrast, brain extracts showed a doublet DMWD signal representing proteins with an apparent MW of approximately 70 kDa. This could suggest that the DMWD protein in brain has a fate or function that is different from that in other DMWD expressing tissues. Differences in molecular environment could contribute to this situation.

Unfortunately the analysis of protein profiles on denaturing gels does not provide any clues about the molecular environment of the folded protein. Therefore we turned to the use of gelfiltration sizing of native protein complexes in extracts of brain, testis and intestine to study any association of DMWD protein with protein partners or other cellular constituents.

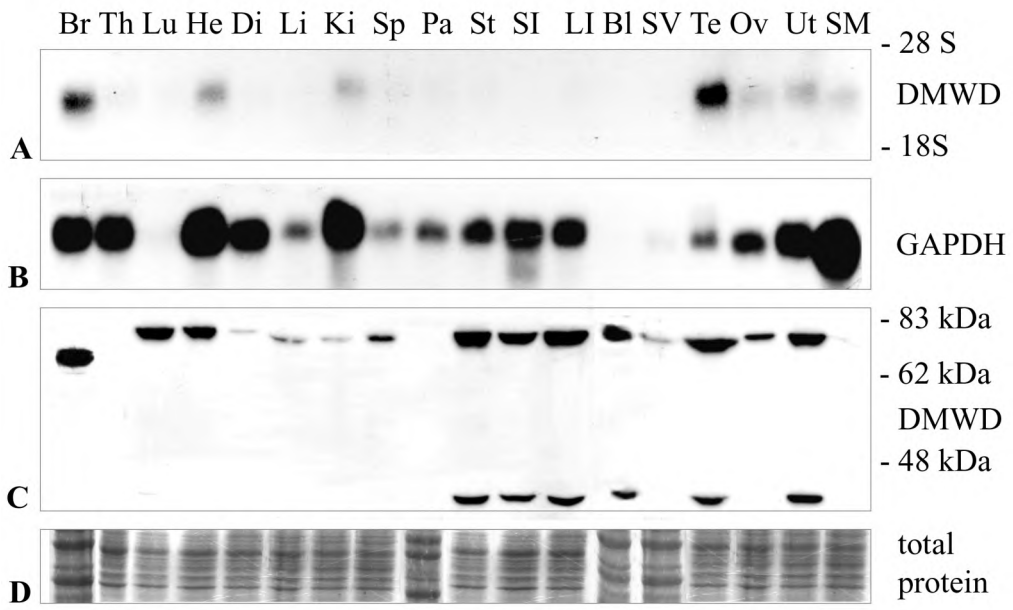


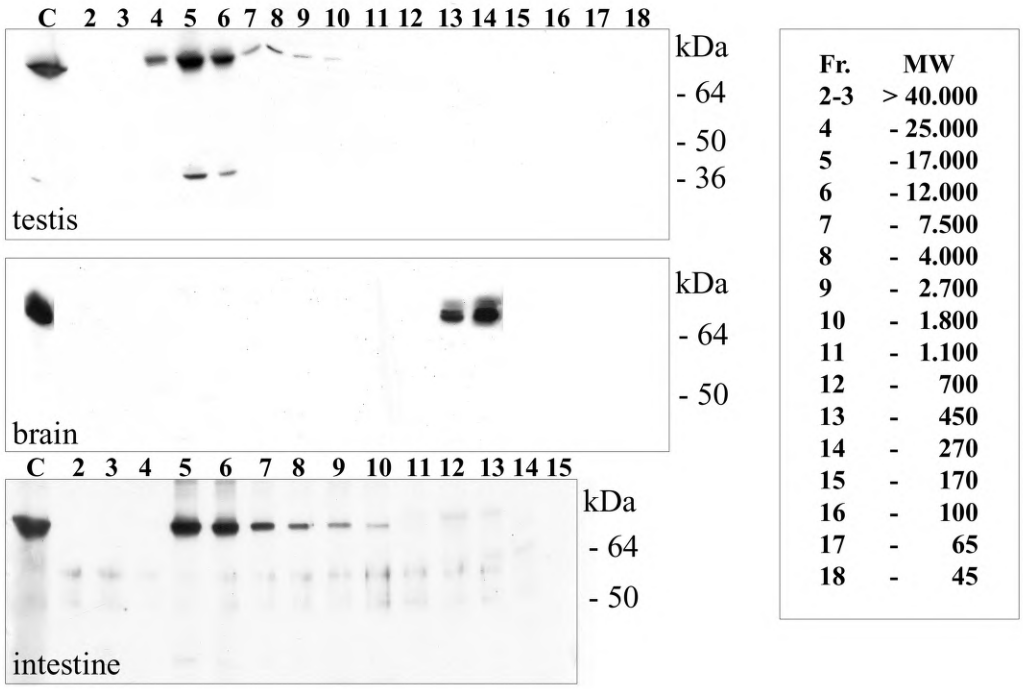

**Fig. 1.** Northern and Western blot analysis of the distribution of DMWD mRNA (A) and protein (C) expression in different tissues. Panel A: DMWD RNA, Panel B: GAPDH RNA, Panel C: DMWD protein, Panel D: total Coomassie stained protein. Panel A and B (18 different tissues) are adapted from [98]. Note that in Panel C (18 different tissues) four distinct DMWD proteins are revealed: A doublet of approximately 70 kDa in brain and two proteins of approximately 78 kDa and 37 kDa in testis and other tissues.
Br: brain, Th: thymus, Lu: lung, Di: diaphragm, Li: liver, Ki: kidney, Sp: spleen, Pa: pancreas, St: stomach, SI: small intestine, LI: large intestine, Bl: bladder, SV: seminal vesicle, Te: testis, Ov: ovary, Ut: Uterus, SM: skeletal muscle.

Proteins in tissue extracts were kept in absence or presence of detergents or under high-salt conditions and were size-fractionated on a Superose 6PC 3.2/30 gelfiltration column which gives optimal fractionation of protein complexes within the 5 to 5000 kDa range (Pharmacia LKB Biotechnology). Western blot analysis was used to reveal presence of DMWD in the different eluate fractions. Fig. 2 shows the results of fractionation of testis (top panel), brain (middle panel) and intestine (bottom panel). Table 1 lists how different treatments have their effect on the elution position of the protein complex(es) in which DMWD is localized.

**Table 1.** Molecular weights (in kDa) of DMWD-containing protein complexes from testis, brain and intestine after treatment of extracts with detergents, high salt or DNAse.

| Treatment | Testis | Brain | Intestine |
|---|---|---|---|
| No treatment | 25.000-12.000 | 450 -170 | 17.000-12.000 |
| NaCl 750 mM | 25.000-7.500 | 270 -170 | 25.000-7.500 |
| Triton 1% | 12.000- 2700 | 270-170 | 12.000-2700 |
| NP40 0.5% | 17.000-7.500 | n.d. | n.d. |
| DNAse | 25.000-7.500 | 450-170 | n.d. |
| SDS 0.5% | 17.000-1800 | n.d. | n.d. |

Shown are the size ranges for DMWD complexes assessed by gelfitration chromatography as described in Fig. 2. Note that most treatments seem to have little effect on the integrity or coherence of structures in which DMWD resides. n.d.: not determined




| Fr. | MW |
|---|---|
| 2-3 | > 40.000 |
| 4 | - 25.000 |
| 5 | - 17.000 |
| 6 | - 12.000 |
| 7 | - 7.500 |
| 8 | - 4.000 |
| 9 | - 2.700 |
| 10 | - 1.800 |
| 11 | - 1.100 |
| 12 | - 700 |
| 13 | - 450 |
| 14 | - 270 |
| 15 | - 170 |
| 16 | - 100 |
| 17 | - 65 |
| 18 | - 45 |

**Fig.2.** Western blot analysis indicating absence or presence of DMWD in different elution fractions of a Superose 6PC column gel filtrate of protein complexes in extracts from testis, brain and intestine. Numbers (2-18) indicated above the top panel correspond to successive (65 µl) elution fractions. To the right, a conversion table is given with size estimates of protein-complexes in the different fractions (molecular weight MW, in kDaltons). Sizing is based on calibration of the column with a mixture of reference proteins with known MWs (see M&M). Sample C is a control sample with DMWD protein (expressed in testis, brain or intestine) in native buffer (no treatment - no gelfiltration). The position of DMWD protein on blot was revealed with the anti-DMWD B13 serum.

The size of the protein complex, in which DMWD is located, appeared clearly dependent on the tissue analyzed. In testis and intestine DMWD was present in protein complexes or particulate matter with a very large MW of approximately 15.000 kDa. In brain DMWD was identified in structures with a smaller apparent MW of approximately 270 kDa. Both types of DMWD-complexes appeared very stable as treatment with detergents like NP40 and SDS or even high salt conditions did not cause a significant shift in their elution profile. In Triton X-100 the size of the protein complex was slightly reduced but remained of large size, 12.000 to 2700 kDa. Also SDS caused a shift in the elution profile but mostly extended the range of fractions in which DMWD protein could be found. This suggests that only a (small) part of the DMWD protein complex was instable in SDS (the MWs of 7.500 to 1800) while leaving the rest intact (MWs of 17.000 to 7.500).
We also checked for presence of DNA in the complex (see sub-chapter yeast two hybrid screening for a rationale) by treating the DMWD complex with DNAse. Again no size alteration was seen, suggesting that the DMWD protein complex is either not directly connected to DNA or that DNA - if present in the complex - is not accessible to nucleolytic attack.

*Yeast two hybrid screening*
To identify any partner proteins that engage in a physical interaction with DMWD we used the LexA-based yeast two hybrid screening procedure. Three different bait plasmids were designed (see Fig. 3).

One vector, the pMW101-DMWD$_{n7-111}$, contained the cDNA segment specifying the proline rich region of the N-terminal part of DMWD and comprises amino acid 7 to 111. The second vector, designated pMW101-DMWD$_{wd192-453}$, contained the cDNA segment specifying the four WD-repeats of DMWD and contained amino acid 192 to 453. The third vector, pMW101-DMWD$_{fl1-650}$ comprises the full-length ORF of the DMWD protein, amino acid 1 to 650. All three vectors were used for transfection of EGY48 yeast cells containing a human fetal brain library (4B) in prey plasmid pJG4. Amongst $10^6$ transformants 3 clones were recovered that showed an interaction with the WD-repeats of DMWD (vector pMW101-DMWD$_{wd192-453}$), 16 clones were retrieved that interacted with the N-terminal part of DMWD (vector pMW101-DMWD $_{n7-111}$) and no clone was identified that represented partners for the full-length protein (vector pMW101-DMWD$_{fl1-650}$). All cDNAs of the positive clones were sequenced and their identity established by BLAST searches. The inserts of the three prey plasmids that were pulled out with the pMW101-DMWD$_{wd192-453}$ vector specified entirely distinct proteins. One, Yme1, appeared to be an ATPase associated with a variety of cellular activities belonging to the so-called AAA-family of proteins [213].

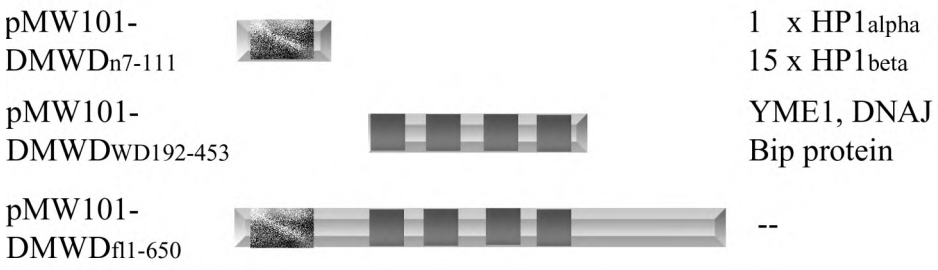



**Fig.3.** Summary of the yeast-two-hybrid screening with different segments of DMWD as bait.
To the left the designations of the vectors used (corresponding to the domain of DMWD) are listed. In the middle an artistic impression of the size and relative orientation of DMWD domains specified by these vectors is given. Names for proteins specified by the different prey plasmids are indicated to the right. The dark spotted areas represent the proline rich parts in the pMW101-DMWD$_{n7-111}$ and pMW101-DMWD$_{fl1-650}$ baits vectors. The dark grey areas represent the four WD-repeats in the pMW101-DMWD$_{wd192-453}$ and the pMW101-DMWD$_{fl1-650}$ vectors.

The other two clones represented the DNAJ heat shock protein [156] and the heat shock protein BiP [120]. All three proteins, Yme1, DNAJ and BiP are known to have chaperone or chaperone-like activity and assist in (re)folding of denatured or partly unfolded proteins. As proteins with these properties have been often found as false positives in yeast-two-hybrid screenings (see for information http://www.fccc.edu/research/labs/golemis/) we abrogated further analysis of these clones.

The 16 positive interactors that were picked up with the pMW101-DMWD $_{n7-111}$ vector originated all from the same family of proteins. One cDNA corresponded to the full-length alpha variant of the human Heterochromatin Protein 1 (HPI$^{Hs}$), the fifteen others corresponded to the cDNA for HP1gamma (see Fig. 3). The results of the yeast-two-hybrid screenings are summarized in Fig.3.
The HPI$^{Hs}\gamma$ protein, found as partner for the DMWD$_{n7-111}$ bait protein, is 42% identical and 58% similar to the Drosophila Heterochromatin protein I (HPI), which is a chromatin binding protein involved in position-effect variegation [47,97]. HPI protein and orthologous proteins in other species are members of the super family of chromo-proteins.

The main shared feature of this class of proteins is the presence of a so-called chromo-domain, which was first identified in HPI and Polycomb [2,119] and later in numerous other proteins as well. The HPI polypeptide also comprises a second domain, the chromo-shadow domain, which is homologous but definitively distinct from the chromo-domain [2]. $HPI^{Hs}\gamma$ and also the independently identified $HPI^{Hs}\alpha$ protein (Fig.3) possess both domains (Fig. 4 and 5). To identify which segment of the HP1 polypeptide was involved in binding to the N-terminus of DMWD, we sequenced all 15 $HPI^{Hs}\gamma$ cDNAs and aligned the regions of overlap (Fig. 4).

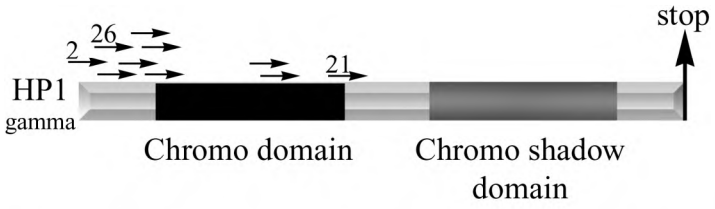



**Fig. 4.** 5'-ends (arrows) of cDNA inserts of ten different Hp1-gamma y2h clones that showed interaction with the $DMWD_{n7\text{-}111}$ bait protein. All inserts contained the stop codon and part of the 3'-UTR. The first dark area correspond to the sequences for the chromo domain the second to the chromo shadow domains in the Hp1 protein. The three arrows (2: $HPI_{fl1\text{-}173}$, 21: $HPI_{77\text{-}173}$ and 26: $HPI_{5\text{-}173}$) with rounded ends indicate the 5'-ends of the inserts in the three cDNAs that were used for further experiments.

Among the fifteen cDNAs inserts ten were unique and five occurred twice (presumably because the fetal brain library was amplified). All ten insert contained the stop codon of HP1 and part of the 3'-UTR. Seven out of ten cDNAs encoded both the chromo- and chromo-shadow domain whereas the 5'-terminal ends of three cDNAs were situated inside the chromo domain sequence, suggesting that it is the chromo-shadow domain or another shared feature with unknown domain specificity (i.e. the grey area in Fig. 4) in the C-terminal part that is involved in the interaction with DMWD.

```
  1          15 16          30 31          45 46          60 61          75
γ MGKKQNGKSKKVEEA ---EPEEFVVEKVLD RRVVNGKVEYFLKWK GFTDADNTWEPEENL DCPELIEAFLNSQKA 72
β MGKKQN--KKKVEEV LEEEEEEYVVEKVLD RRVVKGKVEYLLKWK GFSDEDNTWEPEENL DCPDLIAEFLQSQKT 73
α MGKKT---KRTADSS SSEDEEEYVVEKVLD RRVVKGQVEYLLKWK GFSEEHNTWEPEKNL DCPELISEFMKKYKK 72

  76          90 91          105 106         120 121         135 136         150
γ GKE------KD---G TKRKSLSDSES--DD SKSKKKRDAA-DKPR GFARGLDPERIIGAI DSSGELMFLMKWKDS 135
β AHE------TDKSEG GKRKADSDSEDKGEE SKPKKKKEES-EKPR GFARGLEPERIIGAT DSSGELMFLMKWKNS 141
α MKEGENNKPREKSES NKRK--SNFSNSADD IKSKKKREQSNDIAR GFERGLEPEKIIGAT DSCGDLMFLMKWKDT 145

  151         165 166         180 181         195 196
γ DEADLVLAKEANMKC PQIVIAFYEERLTWH SCP-EDEAQ------ --    173
β DEADLVPAKEANVKC PQVVISFYEERLTWH SYPSEDDDKKDDKN- --    185
α DEADLVLAKEANVKC PQIVIAFYEERLTWH AYP-EDAENKEKETA KS    191
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**Fig. 5** (previous page). Multiple alignment of the three human HPI protein variants, γ: $HPI^{Hs}\gamma$, β: $HPI^{Hs}\beta$ and α: $HPI^{Hs}\alpha$. The HPI proteins are characterized by the presence of a chromo domain at the amino terminus and a chromo-shadow domain at the carboxy-terminus [2]. In Drosophila, the chromo-shadow domain is involved in nuclear targeting and mediates a wide range of protein-protein interactions while the chromo domain is needed for recognition of the methyl marker [59,172,173]. The chromo domain and the chromoshadow domain respectively are marked in bold. The Nuclear Localization Signal (NLS) is underlined.

To verify that genuine interactions between the $DMWD_{n7\text{-}111}$ bait protein and the HP1 protein segment were involved in trans-activation of reporter genes in our yeast colonies we decided to swap inserts between prey and bait plasmids and repeat the screening procedure (see Fig.6). For this test the positive HPI clones 2 ($HPI_{fl1\text{-}173}$), 21 ($HPI_{77\text{-}173}$) and 26 ($HPI_{5\text{-}173}$) (indicated with arrows in Fig. 4) were used. We also included the $DMWD_{wd192\text{-}453}$ and the $DMWD_{fl1\text{-}650}$ inserts as bait with the different HPI cDNAs as prey to see if any of the HPI products would interact with the WD-repeats or the DMWD full-length protein (Fig. 6). As demonstrated in Fig. 6, all three $HPI^{Hs}\gamma$ products: $HPI_{fl1\text{-}173}$, $HPI_{77\text{-}173}$ and $HPI_{5\text{-}173}$ selectively reacted only with the proline-rich $DMWD_{n7\text{-}111}$ protein segment but not with any of the WD-repeats in $DMWD_{wd192\text{-}453}$ or with the full length $DMWD_{fl1\text{-}650}$ protein.

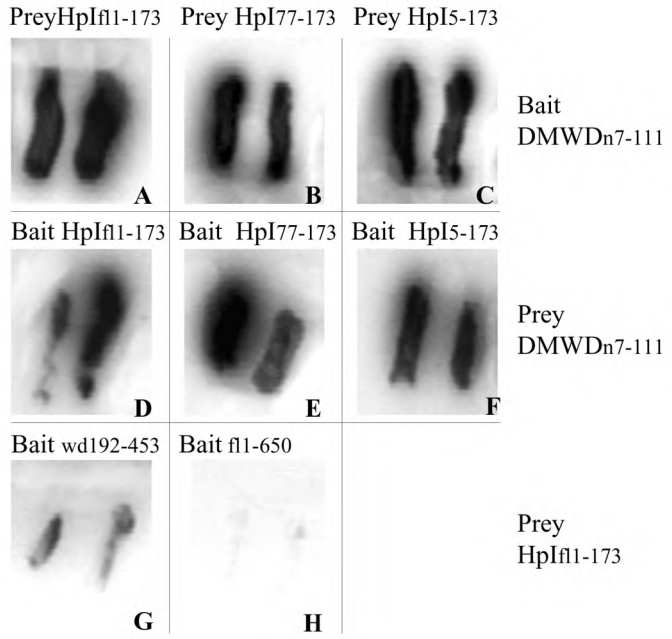


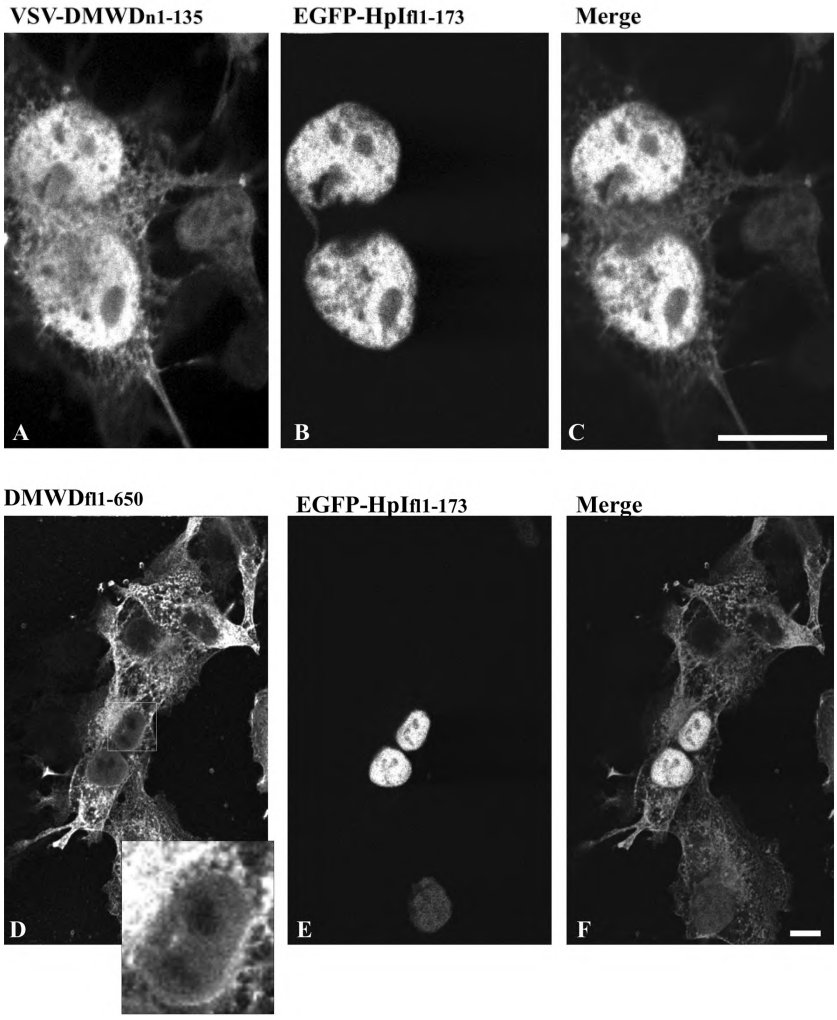

**Fig. 6.** X-gal assay plate for the yeast two hybrid interaction between HPI and DMWD with swapped prey and bait inserts.
Panel A to C: interaction between the HPI clones, $HPI_{fl1\text{-}173}$ (2), $HPI_{77\text{-}173}$ (21) and $HPI_{5\text{-}173}$ (26) and the prey, $DMWD_{n7\text{-}111}$ originally used in the yth-screening. Panel D to F: same with inserts in the bait and prey vectors swapped. Panel G: bait vector $DMWD_{wd192\text{-}453}$ and prey $HP1_{fl1\text{-}173}$. Panel H: bait vector $DMWD_{fl1\text{-}650}$ and prey $HP1_{fl1\text{-}173}$. Note that HP1 interacts with the N-terminal part of DMWD ($DMWD_{n7\text{-}111}$) but not with the WD-repeats ($DMWD_{wd192\text{-}453}$) or the full-length DMWD ($DMWD_{fl1\text{-}650}$) proteins.

In spite of the length differences between proteins specified by plasmids in $HPI_{fl1\text{-}173}$, $HPI_{77\text{-}173}$ and $HPI_{5\text{-}173}$ the DMWD-HP1 binding avidity (as assessed from colony growth rate on leu-plates and beta-galactosidase staining intensity) had approximately the same strength (Fig. 6). This strongly suggests that the entire sequence for the amino acid tract involved in interaction is contained in each of these clones.

The next step was to test if these interactions were also seen if DMWD and HP1 proteins were co-expressed in eucaryotic cell-systems. Therefore, we expressed the three different $HPI^{Hs}\gamma$ segments of clones 2: $HPI_{fl1\text{-}173}$, 21: $HPI_{77\text{-}173}$ and 26: $HPI_{5\text{-}173}$ (Fig. 4) as C-terminally tagged EGFP-fusion proteins in COS1 cells. Simultaneously the N-terminus (amino acid 1-135) of DMWD, tagged with a VSV amino acid segment (pSG8-VSV-$DMWD_{n1\text{-}135}$), was co-tranfected (see Materials and Methods). At 24 hours after transfection protein products with the anticipated MWs (for EGFP-HPI variants in between 32-40 kDa; pSG8-VSV-$DMWD_{n1\text{-}135}$ approximately 17 kDa and $DMWD_{fl1\text{-}650}$ approximately 70 kDa) were already formed as assessed by Western-blot analysis with polyvalent abB13, abH84 and αGFP and monoclonal αVSV antibodies (data not shown). However, when cell extracts were prepared and co-immunoprecipitation assays were performed (see M&Ms for all combinations tested) with the same antibodies abB13, abH84, αGFP and αVSV no interaction between the DMWD proteins ($DMWD_{n1\text{-}135}$ and $DMWD_{fl1\text{-}650}$) and the $HPI^{Hs}\gamma$ proteins (EGFP-$HPI_{fl1\text{-}173}$, EGFP-$HPI_{77\text{-}173}$ and EGFP-$HPI_{5\text{-}173}$) could be detected (data not shown).

Our experiments indicated that there was no problem with the specificity of the antibodies and that sufficient amounts of (VSV-)DMWD and EGFP-HPI proteins were expressed for detection with Western blot analysis. It is possible however that either COS1 cells do not provide the proper environment for DMWD and HP1 proteins to interact or our experimental conditions for cell lysis and incubations did not allow interaction to occur or persist.

Next we analyzed COS-1 cells with protein products from co-transfected EGFP-$HPI_{fl1\text{-}173}$ and pSG8-VSV-$DMWD_{n1\text{-}135}$ or $DMWD_{fl1\text{-}650}$ plasmids with confocal scanning microscopy. The full-length DMWD protein, when expressed in COS1 cells, is located predominantly in the cytoplasm although a small portion of the protein could be detected in the nucleus (see Fig. 7). We also examined if the presence of the full-length HPI protein changed the localization of the full-length DMWD protein but we detected no difference in localization between the singly expressed $DMWD_{fl1\text{-}650}$ or co-expressed (with $HPI_{fl1\text{-}173}$) $DMWD_{fl1\text{-}650}$. In contrast to the full-length DMWD protein, the VSV-tagged $DMWD_{n1\text{-}135}$ protein (pSG8-VSV-$DMWD_{n1\text{-}135}$) was (almost) exclusively located in the nucleus as was the $HPI_{fl1\text{-}173}$ protein (Fig. 7, panel A-C). Both proteins, $DMWD_{n1\text{-}135}$ and $HPI_{fl1\text{-}173}$, are dispersed throughout the nucleoplasm with clear sparing of the nucleoli and show co-localization which was also confirmed by Z-scan analysis through the nucleus (not shown).



To further analyze the localization of the different DMWD and HPI protein we performed computer prediction analysis and especially searched for nuclear localization signals (NLS) and nuclear export signals (NES). NES are short (10-12 amino acids) Leu-rich hydrophobic sequences which were found in several proteins including MAPK kinase 6 [84], protein kinase inhibitor [230] and phospholipase [237].

```
1     MGDCAEIKSQ FRTREGFYKL LPGDATRRSG PTSAQTPAPP QPTQPPPGPA AASGPGAAGP  60
61    ASSPPPAGPG PGPALPAVRL SLVRLGDPDG AGEPPSTPSG LGAGGDRVCF NLGRELYFYP  120
121   GCCRSGSQRS IDLNKPIDKR IYKGTQPTCH DFNQFTAATE TISLLVGFSA GQVQYLDLIK  180
181   KDTSKLFNEE RLIDKTKVTY LKWLPESESL FLASHASGHL YLYNVSHPCT STPPQYSLLK  240
241   QGEGFAVYAA KSKAPRNPLA KWAVGEGPLN EFAFSPDGRH LACVSQDGCL RVFHFDSMLL  300
301   RGLMKSYFGG LLCVCWSPDG RYVVTGGEDD LVTVWSFTEG RVVARGHGHK SWVNAVAFDP  360
361   YTTRAEEAAS ASADGDPSGE EEEPEVTSSD TGAPVSPLPK AGSITYRFGS AGQDTQFCLW 420
421   DLTEDVLSPH PSLARTRTLP GTPGATPPAS GSSRAGETGA GPLPRSLSRS NSLPHPAGGG  480
481   KAGGPNASME PGIPFSIGRF ATLTLQERRD RGAEKEHKRY HSLGNISRGG SGGNSSNDKL  540
541   SGPAPRSRLD PAKVLGTALC PRIHEVPLLE PLVCKKIAQE RLTVLLFLED CIITACQEGL  600
601   ICTWARPGKA FTDEETEAQA GQASWPRSPS KSVVEGISSQ PGSSPSGTVV             650
```

**Fig.8.** Predicted Nuclear Export Signals (NES) in the DMWD protein.
The predicted NES are underlined, all leucines (L) are in bold and all hydrophobic amino acids within the NES are in italics.

Computoral analysis revealed no (obvious) nuclear localizaton signal (NLS) but interestingly two possible nuclear export signals (NES) were predicted (Fig. 8), one at aminoacid 201-212 and one at amino acid 581-590 indicating that the nucleus is not DMWD's main destination.

It is important to note here that all co-immunoprecipitation and microscopical co-localization studies were performed with the intention to test the behaviour of $HPI^{Hs}\gamma$ as a possible partner. Although $HPI^{Hs}\alpha$ behaviour was not directly tested this protein is also known as a protein that exclusively resides in the nucleus [152].

**Fig. 7** (previous page). Confocal scanning microscopical analysis of COS1 cells transfected with DMWD and $HPI^{Hs}\gamma$ cDNAs.
Panel A: COS1 cells expressing pSG8-VSV-DMWDn1-135, detected with monoclonal VSV antibody. Panel B: same COS1 cells expressing EGFP-HPIfl1-173, detected with polyvalent EFGP antibodies. Panel C: merge of both images. Panel D: COS1 cells expressing DMWDfl1-650, detected with polyvalent DMWD antibodies (abB13) an enlargement of the nucleus is showed in the lower right corner. Panel E: Same cells expressing EGFP-HPIfl1-173, detected with polyvalent EFGP antibodies. F. Merge of the two images.
Note that panel C shows nucleoplasmic co-localization of the VSV-DMWDn1-135 protein with the EGFP-HPIfl1-173 protein while no co-localization was detected in panel F (DMWDfl1-650 with EGFP-HPIfl1-173). However, the enlargement in panel D (lower right corner) shows that a small fraction of the full-length DMWD protein is located in the nucleus. Bars represent 10 μm.

## Discussion

*DMWD role in protein complexes*

Here we describe the search for binding partners of the DMWD protein. In different tissues DMWD, when in denatured form, has different appearances. The apparent discrepancy between the DMWD mRNA levels seen on Northern blots and the DMWD proteins levels on Western blots suggest that besides transcriptional mechanisms (post)translational mechanisms may also be involved in DMWD expression regulation.

On Western blots of brain extracts, the protein is presented as a doublet with an apparent molecular weight of 70 kDa. On blots of extracts from testis, intestine and all other DMWD expressing tissues DMWD appeared as a 78 kDa protein, while in some tissues also a (faint) protein product of 37 kDa was seen. All proteins were recognized by DMWD antiserum abB13 and Western blot signals could be completely blocked by the addition of baculovirus-produced native DMWD protein (not shown) so we consider it likely that all products share common epitopes. Most likely the 70 kDa signal represents unmodified DMWD protein as the size corresponds to the predicted MW based on cDNA data. Cell-type dependent post-translational modification, involving phosphorylation, glycosylation or the addition of ubiquitin moieties and/or proteolytic processing may underlie the appearance of products with other molecular weights. Computer analysis predicted seventeen possible serine/threonine phosphorylation sites (11 putative CK2-phospho sites and 6 PKC-phospho sites), two tyrosine phosphorylation sites and several glycosylation sites (not shown). At present we have no data however to distinguish between the various modification possibilities or proteolytic cleavage modes.

Gelfitration experiments showed that DMWD in testis and intestine resides in similar large-sized (approximately 15.000 kDa) and rather robust protein complexes. Although it is difficult to obtain reliable molecular weight estimates for complexes within this size range, we consider this tentative evidence that the DMWD protein may have the same molecular environment, hence the same biological function, in these tissues. In brain however, the DMWD protein seems to be part of a much smaller complex of approximately 270 kDa and because the size of fully denatured DMWD in brain is also distinct, DMWD may have a different role in the CNS. We are currently focussing on the cell type specificity and subcellular location of DMWD in brain to obtain support for this supposition.

*Yth screening*

By identifying multiple binding-partners with known function(s) in the yeast two hybrid-screening assay we had hoped to reveal indirect clues for DMWD function. Unexpectedly, screening of the fetal human cDNA library yielded only candidates for binding to the DMWD-N terminus (segment $DMWD_{n7\text{-}111}$) and the internal $DMWD_{wd192\text{-}453}$ segment but not for the full-length protein although - by definition - both bait segments are represented herein. The most likely explanation for this unexpected observation is that the test system -the yeast cell- does not provide the appropriate biological environment for expression of full-length DMWD in the proper conformation. Possibly the protein has to be processed to expose features of the N-terminal segment or other segments in a manner necessary for partner protein binding. It is of note here that all three candidate partner proteins for association to the $DMWD_{WD192\text{-}453}$ segment, Yme1, DNAJ and BiP have chaperone (-like) properties and are known to bind to incorrectly folded or denatured proteins. Although we did not pursue their candidacy further this observation to us strongly suggest that the internal WD-repeat segment was not properly folded and may need the context of the full protein for proper expression in the yeast system.

Another possibility is that the (potential) protein partner(s) of $DMWD_{fl1-650}$ (also) need to be modified before binding can be established and that the yeast cell does not provide the appropriate environment for these modifications. Support for this latter theory comes from the observation that interaction of CreC (the DMWD homologue in *A. nidulans*) with CreB (a deubiquitinating enzyme) could be demonstrated by functional study in mutants but when interaction was tested in the yeast two hybrid system, no interaction could be detected [134].

Remarkably all sixteen prey clones that interacted with the N-terminal part of DMWD represented members of one protein family, the HPI family of heterochromatin proteins. In humans this family consists of three proteins: $HPI^{Hs}\gamma$ (15 prey clones identified), $HPI^{Hs}\beta$ (no prey clone identified) and $HPI^{Hs}\alpha$ (1 prey clone identified) all are very similar to each other and have their highest similarity in the two chromo-domains (bold amino acids in Fig. 5).
The N-terminal part of DMWD ($DMWD_{n7-111}$) that interacts with $HPI^{Hs}\gamma$ and $HPI^{Hs}\alpha$ has no conspicuous features except that it is rather proline rich (23 prolines within 106 amino acids). Extensive BLAST and FASTA searches did not reveal any homology to known proteins or domains thereof (not shown) so we cannot compare our results to other findings. It is known however, that presence of the amino acid proline is often a critical feature in the primary structure of ligands for engagement in protein-protein interactions [111]. Several protein domains have now been identified that bind to proline rich sequences. The best known is the Src homology 3 (SH3), which is a domain of 50-70 amino acids long and often present in eukaryotic signal transduction and cytoskeletal proteins (see for review [111]). SH3 domains are known to recognize proline rich sequences that have the PXXP motif (X denotes any amino acid) [49,145]. Although the N-terminal part of DMWD has several of these motifs it did not interact with any SH3 containing proteins. Importantly, members of the HPI family of proteins are not known to bind specific to proline rich sequences although Smothers and Henikoff [200] found that the HPI chromo shadow domain binds to a consensus peptide that in most cases starts with a proline.

*HPI protein binding and nuclear role*

Is there a clue in HP1 function that might shed light on the biological role of DMWD?
HPI proteins are believed to function as adaptors, bringing together different proteins in multi-protein complexes and locating them in heterochromatin via protein-protein interactions with the chromo and chromo-shadow domains [2,200,238]. HPI was first identified in Drosophila and in this organism it is involved in a phenomenon known as position effect variegation (PEV) [47,97]. Position-effect variegation is the result of chromosomal rearrangements that place euchromatic genes next to heterochromatin, leading to heritable inactivation of these genes in some cells while in others they remain transcriptional active [135]. Recently, Bannister et al (2001) showed that in the fission yeast *S. Pombe* the HPI protein by it's chromodomain very specifically recognizes a "methyl marker" on lysine 9 of histone H3 and through this recognition the binding occurs [12]. In human it is known that SUV39H1 places a 'methyl marker' on histone H3, which is then recognized by HP1 through its chromo domain [12].
In Drosophila only one HPI polypeptide is known whereas in mammals like human and mouse three HPI like proteins were identified [59,162,188,197,238]. In mice these HPI-like proteins are 98-100% identical to the human proteins, and designated mHPIα, M31 (mMOD1, homologue to $HPI^{Hs}\beta$) and M32 (mMOD2, homologue to $HPI^{Hs}\gamma$) [78,91,125,162,167,238]. Although the variants of HPI are highly conserved and only differ in a few amino acids (see Fig. 5) their location within the nucleus is not always the same. Primary sequence features and the level of phosphorylation seem to be the determining factors for localization behaviour.

For example, mHPIα and mHPIγ are phosphorylated at different phases of the cell cycle, more extensively in mitosis than in interphase, but mHPIβ remains unphosphorylated. This makes that in transfected HeLa cells mHPI isoforms segregate in distinct nuclear domains but in T3T interphase cells HPIα and HPIβ they seem to co-localize [152].
As demonstrated in this study the N-terminal part of DMWD ($DMWD_{n7\text{-}111}$) binds to $HPI^{Hs}\gamma$ most likely via the chromo-shadow domain or the sequence connecting the two domains. These results are consistent with the notion that it is usually the chromo-shadow domain that is dominant in protein-protein interactions [200]. Although we could not demonstrate protein-protein interaction by immuno-pull down, we did show that $DMWD_{n7\text{-}111}$ and the $HPI^{Hs}\gamma$ protein both, independently of each other, are localized in the nucleus. Z-scans through the nucleus of the transfected cells confirmed that both proteins – at least in part - co-localized. Still, it is of note here that functional co-localization of the $DMWD_{n7\text{-}111}$ and the $HPI^{Hs}\gamma$ is strictly not proven, as we did not do any bleaching experiments to test if diffusion behaviour of the two proteins would be similar.
There could be several reasons why we were unable to detect an interaction of DMWD ($DMWD_{n7\text{-}111}$ or full-length) and HPI proteins via co-immuno precipitations. Firstly, as the mHPIγ protein is usually phosphorylated during the cell-cycle [152] it is possible that in the COS1 cells this phosphorylation did not occur with no interaction as a result. It is also conceivable that DMWD should be phosphorylated as well, before an interaction can occur. Secondly, it is possible that the interaction is transient and occurs very rapid and cannot be detected by immuno precipitations, which needs a slow or highly avid interaction to show.
From several studies it appears that HPI proteins have the capacity to interact with proteins with a wide spectrum of different functions and that they can form large macromolecular complexes [125,152]. For this reason it is possible that interaction was not achieved because $HPI^{Hs}\gamma$ may need additional helper proteins that were not available in COS1 cells but where available in yeast. Our gelfiltration experiments showed however that once the interaction has taken place the protein complex in which DMWD resides is very stable.

For DMWD protein to be an interactor for $HPI^{Hs}\gamma$ we anticipated its main location to be within the nucleus. Although this prediction held true for the N-terminal segment of DMWD, the localization of GFP-tagged DMWDfl protein was, however, mainly in the cytoplasm with only a small portion of the protein residing in the nucleus. Conspicuously, neither the N-terminal part $DMWD_{n7\text{-}111}$ nor the full-length DMWD protein did contain any obvious nuclear localization signal. Instead only two putative nuclear export signals (NES) were revealed (Fig. 8), suggesting that the nucleus is not DMWD's main destination. It may therefore be of note here that in a related study (results not shown) we have found that endogenous DMWD protein in neurons is also partly present in the nucleus. Active migration between the cytoplasmic and nuclear compartments can be a bidirectional process, as exemplified by several cytoplasmic proteins which are imported into the nucleus and after assembly or binding to tRNAs or mRNAs are remigrated into the cytosol [66,67,103]. For example karyopherin Kap142p mediates the nuclear import of replication protein A (RPA) and importin β mediates the nuclear import of the fibroblast growth factor receptor [181,239]. Also, for P53, we now know that it is transported into the nucleus along microtubules in a dynein-dependent way [63]. Furthermore, even cell adhesion molecules from the cell cortex can regulate nuclear signaling events. Engagement of the leukocyte integrin LFA-1/αLβ2 leads to binding and subsequent promotion of the nuclear localization of JAB1 (c-Jun coactivator) and ultimately to enhaced activation protein 1 (AP-1) transcriptional activity [17]. It may therefore very well be that cell-type and cell-interaction dependent events, involving assembly with carrier proteins and/or post-translational modification, determine DMWD distribution in cytosol and nucleoplasm.

Finally, another reason to strongly believe in the interaction of DMWD with $HPI^{Hs}\gamma$ is the report of Metzler-Guillemain (2003) that states that $HPI^{Hs}\gamma$ and $HPI^{Hs}\beta$ are concentrated in the sex vesicle during spermatogenesis [150]. Recently we have found that DMWD protein is also located in this vesicle during spermatogenesis [Chapter 3, this thesis]. Which means that both proteins are located in this very specialized structure, giving them the oportunity to interact.

*HPI and disease manifestation in myotonic dystrophy (DM1)*

If DMWD binds to HP1 and has a role in modulating chromatin configuration, could this explain its presumed involvement in disease manifestation in DM1 patients, particularly in the brain and testis features of this disorder? It is tempting to speculate that male sterility in DM1 patients with long CTG-expansions could be determined by abnormal DMWD function. HPI localization in sex vesicles, where obligatory pairing of X and Y chromosomes takes place, is thought to coincide with the beginning of facultative heterochromatin formation. Presumably this has a role in sex-chromosome inactivation [155].

In mammals the XY body formation appears to be essential for pachytene spermatocytes to proceed through meiosis [86]. If in DM patients DMWD expression were to be altered due to CTG-expansion in the neighbouring DMPK gene, it is conceivable, that the formation of the sex vesicle would not proceed correctly and as a result pachytene spermatocytes would also not proceed through meiosis. This would lead to a lower production of sperm and/or complete male sterility.

By analogy with other systems we can also infer weak support for DMWD's presumed role in regulation of chromatin assembly. In a recent report products of the *creC* gene, the homologue of DMWD in *Aspergillus nidulans*, were found to be involved in the transcriptional repression of formation of carbon catabolites. CreC mutants showed derepression in the presence of D-glucose of systems that are normally subject to carbon catabolite repression, hence the repression of the gene(s) is abolished [133,217]. Based on this and further evidence it was proposed that derepression is achieved by binding of CreC (a WD-protein) to CreB (a deubiquitinating enzyme). Through this binding it stabilizes the CreB enzyme [134]. The stabilized CreB enzyme removes the ubiquitin moieties from CreA (a domain DNA binding regulatory protein involved in carbon catabolite repression in *A. nidulans*), which results in repression of transcription of, for instance, the alcA gene [133,134]. If – by analogy - DMWD would have a similar role in transcription regulation in mammals, this could account for many of the symptoms seen in brain and/or testis of DM patients. If connected to metabolic regulation a myriad of possibilities for connection to brain pathology exists (energy depletion, aging etc), but at the moment this pathobiological role remains entirely speculative.

Future research with knockout or knockdown of function in cell and animal model systems is therefore necessary before we can draw more definitive conclusions regarding the role of DMWD in CNS, testis and various other tissues in health and disease.

# Chapter 5

## Four trypsin resistant WD-repeats form the core of the myotonic dystrophy DMWD protein: biochemical and computational evidence

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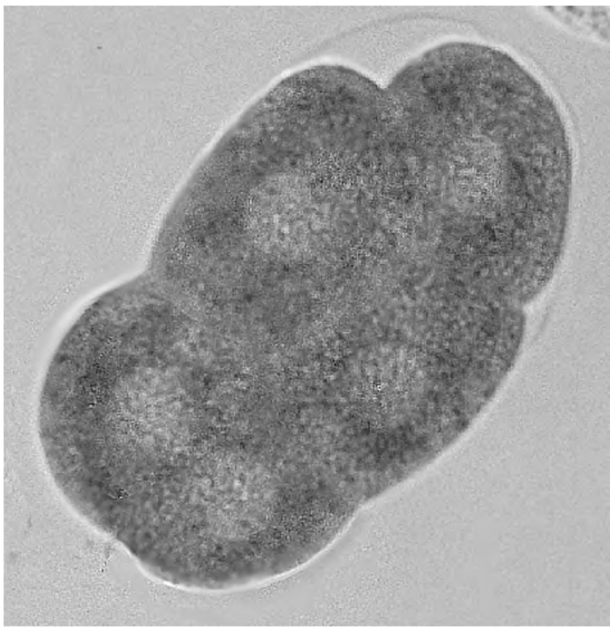

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**Summary**

We used bio-informatics approaches to analyze the WD-repeat segments and other conserved elements in the Myotonic Dystrophy region WD-repeat gene of mouse. From comparison to repeat structures in other WD-proteins and orthologues of the mDMWD gene products in man, *C. elegans, D.Melanogaster, A.thaliana, A.nidulans* and *S.pombe* we predict that the core of the protein comprises four WD-repeats, which are folded in a β propeller-like ring structure. Independent support for the existence of a propeller-ring structure was obtained by biochemical mapping of overlapping trypsin cleavage sites in full-length mDMWD and different GFP-tagged mDMWD segments produced in transfected COS-1 cells. This analysis revealed that the DMWD core is not susceptible to proteolytic attack, like the propeller ring structures in other WD-proteins. For the proline-rich N-terminal domain of DMWD no further structural or functional clues were obtained. The fifth segment with WD-repeat homology in the C-terminal of DMWD might be involved in a regulatory (protein degradation) pathway.

## Introduction

Most proteins are composed of domains, defined polypeptide segments that are conserved during evolution and have a specific catalytic or structural function. There are however, protein domains that share highly conserved amino acid sequence motifs but lack overt functional identity. One of the most common types of domains that possesses clear identity in peptide sequence and 3-D structure but cannot be simply correlated to biochemical function is the WD-repeat. The best-characterized WD-repeat proteins are the G β subunits of heteromeric GTP binding proteins (G-proteins), which consist of seven WD-repeat units and form a dimer with the G γ subunit of large tripartite G-proteins. G-proteins are known to transduce signals across the plasma membrane and are the first WD-repeat protein whose crystal structure was known. The seven WD-repeats of the Gβ subunit protein are arranged in a ring to form a β propeller structure with seven blades. The blades consist of a four-stranded twisted β sheet and binding of the γ subunit to the β subunit is essential for correct folding. [123,204,224]. Detailed structural analysis has revealed that one WD-repeat sequence is not precisely (linearly) equivalent to one propeller blade. Each blade is constructed of the first three strands of one repeat (strand a – c) and the last strand in the next repeat (strand d), an arrangement needed to keep the ring shut [199]. It is thought that every protein with WD-repeats forms this kind of structure, which places all WD-repeat proteins into one large structural family. To make a full-circular structure, four or more repeats in one collinear arrangement are needed, although an alternative possibility would be that proteins with just two or three WD-repeats form dimers and in this way form the rigid ring structure. However, until now no proteins with this latter arrangement were discovered. It is of note here that WD-repeat containing proteins are not the only proteins to form the β propeller ring structure, as cytochrome C [57] and matrix metalloproteinase MMP-1 [127], proteins that share no obvious sequence similarity to WD-repeat proteins, also form these type of structures. Each individual WD-repeat has a few characteristic features. Each comprises a 44-60 amino acid residue sequence that usually contains a GH dipeptide at positions 11-24 from its N-terminus and a C-terminal WD dipeptide. The core in between the GH and the WD is conserved within the protein. There are two variable regions, the first is located just before the GH dipeptide and the second is situated between the a and the b strand (a-b loop; Fig. 1). These variable regions are conserved between orthologous and homologous proteins of different species. The b-c loop is very conserved and consists mostly of three amino acids, the aspartate residue is present in 80% of the repeats but the other residues can be any amino acid. [158,199,222].
WD-repeat rich segments provide to proteins an interface for association to more than one protein partner. Possible binding sites are the top and the bottom of the β propeller ring but also the outside of the propeller (which mostly consists of the d β strands) can interact with proteins. Actually, for some WD-repeat proteins it has already been demonstrated that they can associate simultaneously with multiple binding partners. G β for example interacts with G γ and simultaneously with one of at least 15 other proteins [199]. Another example is the yeast transcriptional repressor TUP1, a protein with 7 WD-repeats. This protein can repress the transcription of several genes by interacting with different proteins, which in turn are bound to DNA sequences. For example α2, a homeo-domain protein, was found to bind directly to TUP1 via its WD-repeats. For this interaction to occur the d-a loops of all WD-repeats are important, as point mutations in these areas prevented α2 binding [116,117].

In humans several inheritable syndromes are thought to be the result of mutant WD-repeat proteins. Recently Tullio-Pelet et al (2000) [220] found that the triple-A syndrome (also known as Allgrove syndrome) was caused by a mutant WD-repeat protein, ALADIN (for alacrima-achalasia-adrenal insufficiency neurologic disorder).

Patients that suffered from this syndrome all lacked the full-length ALADIN protein [220]. Another syndrome known as the Cockayne syndrome, which is a DNA repair disorder, is also caused by a mutant WD-repeat protein called CSA [87]. Mutations in the Cockayne syndrome A or B gene (CSA or CSB) result in defective Transcription-Coupled Repair (TCR). CSA is required for efficient repair during the elongation stages of RNA polymerase II transcription [219] and the translocation of CSA to the nuclear matrix after DNA damage is depended on the Cockayne syndrome B protein [106]. Unfortunately, the mutations and clinical symptoms seen in diseases known to be caused by WD-repeat proteins did until now not reveal any clues about the (general) biological functions of these WD-repeats.
In this study we focus on the protein product(s) of the DMWD (Myotonic Dystrophy region WD-repeat gene). DMWD is a gene that in both humans and mice is located just upstream of the Myotonic Dystrophy Protein Kinase gene (DMPK). A peculiar type of mutation, an expansion of a triplet repeat $(CTG)_n$, in the 3'-UTR of this gene in humans, causes Myotonic Dystrophy (DM). DM is a multisystemic disorder and one of the current hypotheses about the disease manifestation is that the triplet repeat expansion causes chromatin distortion, which not only affects the DMPK gene but might also have an effect on neighbouring genes like DMWD (reviewed in [71]). We provide here bio- computational evidence that the name DMWD has been rightfully assigned, as the mouse DMWD protein contains four WD-repeats, which are properly arranged for forming a propeller ring. Moreover, biochemical evidence is provided that the repeat-segment in the mouse DMWD proteins is indeed folded like a genuine β propeller structure with high resistance against trypsin protease activity. A discussion is provided on how these characteristic features are embedded in the complete protein structure, in which a proline rich N-terminal domain and a (fifth) repeat domain with WD-homology in the C-terminus portion of the protein can also be distinguished.

## Materials and methods

*Generation of eukaryotic DMWD expression vectors*
The plasmid pSG8-DMWDfl was constructed by sub-cloning a 2100 bp segment spanning the entire open reading frame of DMWD cDNA into the eukaryotic expression vector pSG8ΔEcoRI. The pSG8ΔEcoRI vector is based on pSG5 [70] and has an extended multiple cloning site (inserted between EcoRI/BglII, containing 6 unique restriction sites, EcoRV, HinDIII, ClaI, SacI, XhoI and DraII). The pSG8-$DMWD_{fl}$ vector was obtained by linking two EcoRI sites to the complete ORF of DMWD, and cloning this fragment in frame into the pSG8ΔEcoRI vector (pSG8-$DMWD_{fl}$).
Plasmid pSG8-$DMWD_{129\text{-}650}$-EGFP was constructed by sub cloning a 1600 bp cDNA segment, encoding the polypeptide segment between amino acid positions 129 - 650(end) of the DMWD protein, into the eukaryotic expression tag vector pSG8-NotI-EGFP-HIS (kind gift of Dr. A.M. van den Maagdenberg). This plasmid specifies a chimeric protein consisting of the WD-repeat segment of DMWD with a C-terminal GFP tag.
To generate the plasmid, the 1600 bp cDNA segment flanked with NotI sites was PCR-amplified with primers JW12WD.for (5′-AT-AAG-AAT-GCG-GCC-GCC-ATG-CGG-TCC-ATC-GAT-CTC-AAC-AAG-3′) and JW10N9.rev (5′-AT-AAG-AAT-GCG-GCC-GCC-ATG-GGC-GAC-TGC-GCG-G-3′) on pSG8-$DMWD_{fl}$. This resulting fragment was cleaved with NotI and inserted into the unique Not1 site of the pSG8-Not1-EGFP-HIS vector. Restriction enzyme digestion and DNA sequencing identified plasmids containing the insert in the correct orientation.

Chimeric plasmid pSG8-DMWD$_{1-135}$ -EGFP was constructed by sub cloning a 450 bp DMWD cDNA segment, containing the N-terminal segment of DMWD (amino acid 1 – 135) in front of the GFP-His tag. Therefore, the pertinent cDNA fragment was PCR amplified with primers JWN9.for (5′-AT-AAG-AAT-GCG-GCC-GCC-ATG-GGC-GAC-TGC-GCG-G-3′) and JW11pro.rev (5′-A-TAA-GAA-TGC-GGC-CGC-CTT-GTT-GAG-ATC-GAT-GGA-CCG-3′) on pSG8-DMWD$_{fl}$ as a template. Subsequently, the resulting DNA fragment was cleaved with NotI and ligated into Not1 linearized pSG8-Not1-EGFP-HIS vector DNA. Plasmids containing the insert in the correct orientation were identified by restriction enzyme digestion, DNA sequencing was performed, and the nature of the protein products was controlled by performing transfection experiments.

*DMWD protein expression and proteolytic digestion assay*

Cos-1 cells were transfected with the different pSG8-based expression vectors by using the DEAE-dextran method. Cells were grown in 10% FCS DMEM medium until 60-70% confluency, in 9 cm culture disks. On the day of transfection fresh medium was given two-three hours before start of the experiment. Transfection started with washing the cells with serum free medium (Optimem), then the DNA/DEAE-dextran mixture (5-10 ug DNA, 9 µl cloroquine (2 mM in MQ), 300 µl dextran (1 mg/ml) and 5.7 ml Optimem) was gently placed on top of the cells. After incubating for two hours at 37°C the DNA/dextran mixture was taken off and cells were shocked for 2 min. at RT with 10% DMSO in PBS. Next, the DMSO solution was replaced with 10% FCS DMEM medium and the cells were incubated at 37°C. Two to four hours later the medium was again substituted with fresh medium and the cells were incubated o/n at 37°C. At time point 20- 24 hours after DMSO treatment the transfected cells were placed on ice, scraped of the disks and diluted in 0.5 ml ice-cold RIPA buffer (20mM Hepes, 150 mM NaCl, 1% Triton, 0.5% DOC (Sodium deoxy cholate), 0.05% SDS) with protease inhibitors (Hoffmann - La Roche Ltd, Swiss). Samples were vortexed for 1-2 minutes, centrifuged (at 13.000 rpm) in an eppendorf centrifuge at 4°C for 15 min and supernatants were transferred to a new tube and kept frozen at –20°C until further use. The total protein concentration of each sample was determined and an amount of lysate with the equivalent of 40 µg protein was used in each trypsin digestion assay. To this end, two different incubation protocols were followed; either different concentrations of L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin (final concentration 3.4 nM-33.6 µM) was added to the protein samples in a total volume of 20 µl and incubated at 37°C for 10 min, or 2 µl of L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated Trypsin (42 µM) was added to the protein samples in a total volume of 20 µl (final concentration of trypsin. 4.2 µM) and incubated at 37°C for 2.5 up to 60 minutes. Digested samples were immediately diluted with an equal volume 2-fold concentrated SDS-sample buffer (200 mM Tris-HCl, pH 6.8, 400 mM dithiothreitol, 8% SDS, 0.4% bromophenol blue, 40% glycerol) and kept on ice until all samples were processed. Subsequently, digests were resolved on PAA-gels and used for Western blot assays.

*Western blot analyzes of intact DMWD and proteolytic breakdown products*

Trypsin digested proteins (20-25 µg) were resolved by electrophoresis on a 15% w/v SDS-polyacrylamide gel and subsequently transferred onto nitrocellulose membranes (Schleicher and Schuell, Keene, NH). Membranes were blocked in 5% skimmed milk in Phosphate Buffered Saline (PBS) with 0.05% Tween-20 (PBST) for minimal an hour at RT. Antibodies were diluted in fresh blocking solution (anti-DMWD abB13 (1:5000) and anti-GFP (1:10,000) [37] and incubated with the blot membrane o/n at 4°C on a rotating platform.

The blots were washed with PBST and incubated with peroxidase-conjugated goat anti-rabbit immunoglobins (Pierce, Rockford, IL) for minimal an hour at RT on a rotating platform. Subsequently the blots were washed with PBST (twice) and PBS (once) and the labelled protein bands were visualized using freshly prepared chemiluminescent substrate (100 mM Tris-HCL, pH 8.5, 1.25 mM p-coumaric acid (Sigma), 0.2 mM luminol (Sigma), 0.009% $H_2O_2$) and exposing the blots to Kodak X-omat film.

*DMWD antibody generation (abB13)*

AbB13: A partial DMWD construct (amino acid 114 - 650) was created by the use of PCR with primer pat1 5′-GGA GAA TTC GAG CTT TAT TTC TAC CCC-3′ (nucleotide position 343 to 361) and pat2 5′-AGC GAA TTC TTA TCA CAC CAC AGT GCC-3′ (nucleotide position 1935 to 1953) on mouse cDNA clone 9.1 [99] (accession numberZ38011-Z38013 and Z38015). This PCR product was digested with EcoRI and ligated in frame into the EcoRI site of the pAr (delta EcoRI)(59/60) expression vector [18,209]. Next, the pArDMWD vector was introduced into E.Coli BL21 (DE3)pLysS (Stratagene, California, USA) and induced with 0.33 mM IPTG (isopropylthio-β-D-galactosidase). The integrity of the induced protein (~ 60kDa) was checked by analysing a small protein sample on a 10% w/v SDS-polyacrylamide gel and the remainder was run on a large 10% w/v SDS-polyacrylamide gel for preparative purification. Protein with the correct size was excised from the gel and the protein was retrieved by electro elution in a Biotrap BT100 apparatus (Schleicher & Schuell) according to manufacturer's protocols. The protein concentration was determined by the Lowry procedure according to Peterson [169] and protein purity was checked again by analysis on a 10% w/v SDS-polyacrylamide gel. A rabbit was immunized once with 400 µg of the purified DMWD protein in a 1:1 v/v ratio with incomplete Freund's adjuvant and three times with 300µg purified DMWD protein, also in a 1:1 ratio. Two months later, after reactivity of the serum was checked with a Western blot on which the DMWD protein was blotted the rabbit was bled and the serum collected and affinity- purified over a column in which the truncated DMWD protein was bound to a matrix (Affi-gel10, Bio-Rad) in 0.1 M MOPS buffer according to the manufacturer's protocols. Purified antibody (designated abB13) was kept at –20 and –80°C.

*Blockmaker, alignments, motif- and WD-repeat search*

The program blockmaker at www.blocks.fhcrc.org was used for defining the conserved blocks in DMWD and his orthologues. Blockmaker is designed to find blocks in a group of related protein sequences. Blocks are short multiple aligned ungapped segments corresponding to the most highly conserved regions of proteins. Typically, a group of proteins has more than one region in common and their relationship is represented as a series of blocks separated by unaligned regions. The minimum amount of sequences is 2 and the maximum is 250, the more sequences you give the more effective this program is. For Blockmaker 7 sequences were used to do the searches: mouseDMWD (SwissProt accession no. Q08274), human DMWD (SwissProt accession no. Q09019), *C. elegans* C08B6.7 (Genbank accession no. Z72502), *Aspergilus nidulans* CreC (Genbank accession no. AF136452), *Schizosaccharomyces pombe* YDE3 (SwissProt accession no. Q10437), *Arabidopsis thaliana* F4P12.90 Genbank accession no. T45879), CG6420 gene product *Drosophila melanogaster* (Genbank accession no. AAF56618.1).

The WD-repeat search was done at http://bmerc-www.bu.edu/wdrepeat/ with the DMWD mouse and CreC sequences. Motif searches were done at http://motif.genome.ad.jp/ with all seven DMWD sequences. All available databases: PROSITE Pattern, PROSITE Profile, BLOCKS, ProDom, PRINTS and Pfam were searched.

## Results

*WD-repeat arrangement in DMWD*

Figure 1 shows the typical arrangement of characteristic amino acid stretches in the consensus WD-repeat.

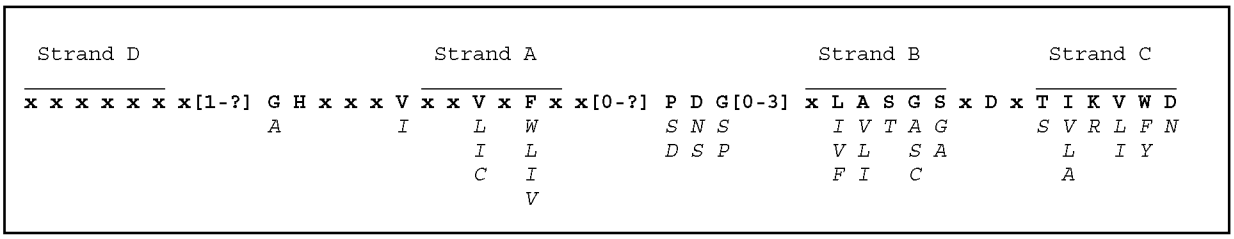



**Fig. 1.** Arrangement of amino acid sequences in different segments of an archetypal (consensus) WD-repeat.
x signifies positions where any amino acid can be found. [0-?] indicates the allowed range of the preceding symbol. Below the sequence in italics, alternative amino acid(s) are specified that are most frequently found in that position. The tertiary structure of the WD-repeat structure in the Gβ subunit of G-proteins has been determined. Based on this model predictions on β strands are shown as over-lines above the different amino acids with "a" being the strand closest to the central pore and "d" being the strand closest to the external surface of the folded protein (http://bmerc-www.bu.edu /wdrepeat/)

To confirm the earlier predicted characteristics of the DMWD protein [98] we took the updated consensus for WD-repeats and conducted a more stringent WD-repeat search at http://bmerc-www.bu.edu/wdrepeat/ based on the predicted amino acid sequence of the mouseDMWD sequence. This classified the DMWD gene product as a four WD-repeat protein, although five WD-repeat like areas were recognized. Typically, WD-repeats are conserved not only with respect to the type of side chain but also in their inter-WD-repeat spacing (reviewed in:[158,199,222]). The average distance between pairs of WD-repeat elements in the area of repeat elements one to four was 20.3 amino acids but the length of the loop between the fourth and fifth WD-repeat was 155 amino acids (Table 1). The probability score for DMWD being a four WD-repeat protein was 0.999 whereas the probability score for DMWD as a five WD-repeat protein was only 0.001. This makes it unlikely that the fifth domain in DMWD serves as a genuine WD-repeat segment and participates in the formation of a propeller structure.

**Table 1.** Inter-repeat distance of mDMWD and CreC

| Putative WD-repeat | Distance between putative WD-repeats in DMWD | Distance between putative WD-repeats in CreC | Ratio repeat distance to average spacing 1-4, DMWD | Ratio repeat distance to average spacing 1-4, CreC |
|---|---|---|---|---|
| 1 and 2 | 39 | 37 | 1.9 | 2.8 |
| 2 and 3 | 11 | 1 | 0.5 | 0.1 |
| 3 and 4 | 11 | 1 | 0.5 | 0.1 |
| 4 and 5 | 155 | 70 | 7.6 | 5.4 |
| Average 1-4 | 20.3 | 13 | 1 | 1 |

Additional indirect evidence came from comparison to the published sequence of the putative DMWD homologue in *Aspergilus nidulans*, CreC [217]. Todd and co-workers originally had suggested that CreC has five WD-repeats. Reanalysis of the amino acid arrangement of segments in the CreC protein initially confirmed this prediction. More careful analysis, however, revealed that also in this case the spacing between the fourth and fifth WD-repeat was unusually large. Whereas the average spacing between repeats in area of the first four repeats was 13 amino acids the length of the loop between repeats four and five was 70 amino acids (Table 1). This means, although the situation is less dramatic than in DMWD, that this area is still five-folds longer than any of the other inter-repeat loop segments in the protein. The probability score for CreC being built as a structure with four WD-repeats was 1.000 and with 5 WD-repeats was 0.000.

*Conserved areas in DMWD*

Another way of predicting features in the DMWD structure is to try to include all conserved areas in a comparison of all known DMWD homologous proteins across species. In this manner attention was diverted from the WD-repeat segments proper, in which conservation was not very strict (Fig. 1). We searched all available databases for full-length sequences of mouseDMWD orthologues and identified six candidates: human DMWD (SwissProt accession no. Q09019), *C. elegans* C08B6.7 (Genbank accession no. Z72502), *Aspergilus nidulans* CreC (Genbank accession no. AF136452), *Schizosaccharomyces pombe* YDE3 (SwissProt accession no. Q10437), *Arabidopsis thaliana* F4P12.90 Genbank accession no. T45879), CG6420 gene product *Drosophila melanogaster* (Genbank accession no. AAF56618.1). A partial sequence of a possible DMWD homologue in *Xenopus* was also found but we were unable to extend this small fragment into a full-length protein. This putative protein was therefore not included in our further analyzes.

To search for blocks of conserved amino acids in related proteins we used the program blockmaker at *www.blocks.fhcrc.org.* Blocks in this program, were defined as short multiple aligned ungapped segments corresponding to the most highly conserved regions of proteins. Typically, if proteins are related they are represented as a series of blocks separated by unaligned regions. Fig. 2 shows the conserved areas of all six putative DMWD orthologues (including CreC) and mouseDMWD. As anticipated, although comparison was now not biased towards any particular sequence feature, in this analysis again the five blocks appeared that corresponded with the five WD-repeat like areas originally recognized in mouseDMWD. The borders of the conserved areas, however, were not entirely congruent with those of the WD-repeats because the blockmaker program only picks up the segments with the highest homology.

Beside this we performed a WD-repeat search at http://bmerc-www.bu.edu/wdrepeat/ based on the predicted amino acid sequence of all six putative DMWD homologous proteins. All showed highest probability scores for the 4-WD-repeat protein (Table 2).

We also ran a computer search to check for homology of the N and /or the C terminal part to any other known protein motif. The N-terminal part of mouseDMWD (amino acid 31 to 76) shows the strongest homology to synapsin proteins but this homology could not be confirmed for any of the other DMWD orthologues.

```
                                area 1 width = 38
     A.thaliana                228 KDGSVNNRQDRCTSISWVPGGDGAFVVAHADGNLYNKD
A.nidulans CreC                280 INKNGIINSSPVTHIKWIPGSENFFIAAHENGQLVVYD
      C.elegans                218 YNEDRYIEKTSVTCIRWLPGDSNIFLASYVSGNLYVYD
 D. melanogaster               242 FNEERLIDKTKVTCLKWLPNSPHLFLAAHASGHLYLYN
     Human DMWD                 83 FNEERLIDKTKVTYLKWLPESESLFLASHASGHLYLYN
     Mouse DMWD                187 FNEERLIDKTKVTYLKWLPESESLFLASHASGHLYLYN
        S.pombe                233 FNKNGQLNSSSVTAIKWVAGKDSQFLVSFRNGWLVLYD

                                area 2 width = 40
     A.thaliana  (  41)        307 IAFSNDGAHLATVGRDGYLRIFDFLTQKLVCGGKSYYGAL
A.nidulans CreC  (  55)        373 FAFSPDHRHLAVVLEDGTLRLMDYLQEEVLDVFRSYYGGF
      C.elegans  (  55)        311 SFSGSDGKMMATVSHDGFLRIFNYHAQELLAVMKSYFGGL
D. melanogaster  (  49)        329 FCFSPCGSHLAVVSQDGFLRVFHYDTMELLGIARSYFGGF
     Human DMWD  (  47)        168 FAFSPDGRHLACVSQDACLRVFHFDSMLLRGLMKSYFGGL
     Mouse DMWD  (  47)        272 FAFSPDGRHLACVSQDGCLRVFHFDSMLLRGLMKSYFGGL
        S.pombe  (  57)        328 FCFSPDYQYLALVSERGTLKLFDFVKEHVLDVFHSYFAGL

                                area 3, width = 43
     A.thaliana  (   5)        352 SMDGKYILTGGEDDLVQVWSMEDRKVVAWGEGHNSWVSGVAFD
A.nidulans CreC  (   5)        418 SPDGKYIVTGGQDDLVTIWSLPERKIIARCQGHDSWVSAVAFD
      C.elegans  (   5)        356 SPDAKLIATGGEDDLLTVYSVAEKRVVCRGQAHKSWVSQVKFD
D. melanogaster  (   5)        374 SPDGKYIVVGGEDDLVTVWSLHERRVVARGQGHRSWVSVVAFD
     Human DMWD  (   5)        213 SPDGRYVVTGGEDDLVTVWSFTEGRVVARGHGHKSWVNAVAFD
     Mouse DMWD  (   5)        317 SPDGRYVVTGGEDDLVTVWSFTEGRVVARGHGHKSWVNAVAFD
        S.pombe  (   5)        373 SPDGKFIAIGGKDDLVSIYSFPLRKLVARCQGHKSWVTDVIFD

                                area 4, width = 16
     A.thaliana  (  16)        411 YRFGSVGQDTQLLLWD
A.nidulans CreC  (   8)        469 YRIGSVGDDCNLLLWD
      C.elegans  (  74)        473 YRIGSVGHDTFLCLWD
D. melanogaster  (  76)        493 YRLGSVSQDTQICLWD
     Human DMWD  (  52)        308 YRFGSAGQDTQFCLWD
     Mouse DMWD  (  46)        406 YRFGSAGQDTQFCLWD
   S.pombe YDE3  (   8)        424 YRIASVGLDRKLLLWD

                                area 5, width = 41
     A.thaliana  (  46)        473 PKLSPIIAHRVHTEPLSGLMFTQESVVTACREGHIKIWTRP
A.nidulans CreC  (  72)        557 ALLPPIMSKAVGEDPICWLGFQEDTIMTSSLEGHIRTWDRP
      C.elegans  ( 147)        636 PMIEPLMCKKVSHDRLTVLEFREDCVVTACQEGYICTWGRP
D. melanogaster  ( 337)        846 PLLEPLVCKKIAHERLTALIFREDCFLTACQDGFIYTWARP
     Human DMWD  ( 146)        470 PLLEPLVCKKIAQERLTVLLFLEDCIITACQEGLICTWPRP
     Mouse DMWD  ( 145)        567 PLLEPLVCKKIAQERLTVLLFLEDCIITACQEGLICTWARP
   S.pombe YDE3  (  63)        503 PVISPITIYDVDDSPLSSVFFDPDCMITCATNGRIRTWQRP
```

**Fig. 2.** Alignment of conserved blocks in six different putative orthologues and the mouseDMWD itself. MouseDMWD (SwissProt accession no. Q08274), human DMWD (SwissProt accession no. Q09019), *C. elegans* C08B6.7 (Genbank accession no. Z72502), *Aspergilus nidulans* CreC (Genbank accession no. AF136452), *Schizosaccharomyces pombe* YDE3 (SwissProt accession no. Q10437), *Arabidopsis thaliana* T2N18.8 Genbank accession no. Z72502), CG6420 gene product *Drosophila melanogaster* (Genbank accession no. AAF56618.1). In the first column the name of the species in which the homologue occurs is given, the second column specifies the number of amino acids that separates any conserved block from the last amino acid in the next preceding conserved block. In the third column the position of the amino acid at which the block homology starts in the actual protein is given and in the last column the amino acid sequences of the different blocks are aligned.

**Table 2.** Probability scores of DMWD homologous proteins.

| | *A. thaliana* | *A. nidulans (CreC)* | *C. elegans* | *D. melano-gaster* | Human | Mouse | *S. pompe* |
|---|---|---|---|---|---|---|---|
| 4 WD-repeat | 1.000 | 1.000 | 0.889 | 0.728 | 0.994 | 0.999 | 1.000 |
| 5 WD-repeat | 0.000 | 0.000 | 0.111 | 0.118 | 0.006 | 0.001 | 0.000 |
| Generic | 0.000 | 0.000 | 0.000 | 0.154 | 0.000 | 0.000 | 0.000 |

Interestingly, two other motifs were more often found: the so-called Tub motif and the Rhodopsin-like GPCR superfamily signature. The Tub motif was discovered in Tub and Tub-like proteins and these proteins play an important role in maintenance and function of neuronal cells during development and post-differentiation [95]. The Tub motif was found in six out of the seven DMWD (homologous) proteins (not in *Aspergilus nidulans*) with probability scores between 1114 (human) and 1011 (*Arabidopsis thaliana*). The other motif: the Rhodopsin-like GPCR superfamily signature, was identified in all DMWD orthologues but the motif had probability scores between 1001 and 1027, close to 1000, and therefore has a very low chance of being a true functional motif.
In the C-terminal part (as shown in Fig. 2) we identified again the block with homology to WD-repeats but no other conserved elements.

*DMWD regions with high resistance and vulnerability against trypsin digestion*
WD-repeats fold into β propeller like structures that are highly resistant or inert to trypsin cleavage [61]. To analyze if the mouse DMWD protein is also folded in the same manner and if the predicted four or all five WD-repeat-like elements were needed to yield the proper configuration we tested the protein protease resistance, in a trypsin-cleavage assay. To this end, eukaryotic expression plasmids specifying three different DMWD protein products were transfected into COS-1 cells: (i) pSG8-DMWDfl, which produces a 650 amino acids full-length DMWD protein, (ii) pSG8-DMWD$_{129\text{-}650}$-EGFP which produces a chimeric protein of 521 amino acids of the WD-repeat segment of DMWD and full-length EGFP fused at its C-terminal end and (iii) pSG8-DMWD$_{1\text{-}135}$ –EGFP, which encodes a polypeptide consisting of the first 135 N-terminal amino acids of DMWD, fused to EGFP at its C-terminus. All three vectors are based on the pSG8-vector, of which the two partial DMWD products contain an EGFP sequence at there C-terminal, while the DMWDfl is used without any additional protein tag. EGFP-tagging was chosen because the GFP-moiety is relatively inert to trypsin cleavage and therefore serves as an easily identifiable demarcation element.
Proteins produced in total COS-1 cell lysates were digested with trypsin and proteolytic breakdown products were analyzed by Western blot analysis and detection with anti-GFP or abB13 (anti-DMWD). Fig. 3 shows the typical Western-blot pattern of mouseDMWDfl products after digestion with increasing amounts of trypsin for a fixed period of time (30 minutes). The two bands seen in lane 1 are both DMWD products. The slowest migrating protein product is the full-length DMWD. The faster migrating component is most likely a breakdown product, as its concentration increases in the presence of trypsin.

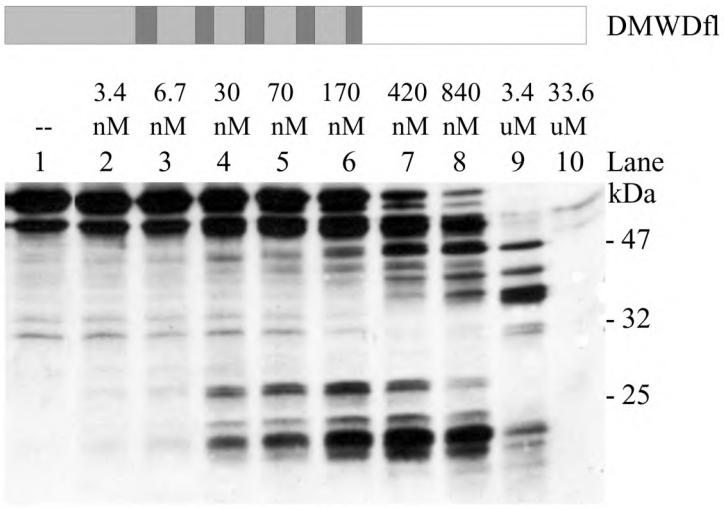



**Fig. 3.** Western blot of trypsin cleavage products of DMWDfl produced in COS-1 cells.
DMWDfl protein products in transfected COS-1 cell lysates were digested with increasing trypsin concentration (3.4 nM lane 2 – 33.6 uM lane 10) for 30 min at 37°C. After digestion protein products were detected with polyvalent abB13. Lane 1 is control DMWDfl product (same total lysate protein concentration as in other lanes) left at 37°C for 30 minutes in the absence of trypsin.

In Fig. 4 all putative trypsin cleavage sites in the linear mDMWD protein sequence are indicated in bold. The specific pattern of proteolytic products visible in lanes 5-10 of Fig. 3 could only be explained if we assumed that some sites in DMWD were more prone to digestion with trypsin than others. The signal intensity of $DMWD_{fl}$ and its breakdown products remained relatively unaltered at low concentrations of trypsin (lanes 1-8). At higher concentrations of trypsin (lanes 7-9) the molecular weights of the antigenic mDMWD breakdown products tended to cluster into two size classes, 40-60 kDa and 10-20 kDa.

```
1    MGDCAEIKSQ FRTREGFYKL LPGDATRRSG PTSAQTPAPP QPTQPPPGPA AASGPGAAGP 60
61   ASSPPPAGPG PGPALPAVRL SLVRLGDPDG AGEPPSTPSG LGAGGDRVCF NLGRELYFYP 120
121  GCCRSGSQRS IDLNKPIDKR IYKGTQPTCH DFNQFTAATE TISLLVGFSA GQVQYLDLIK 180
181  KDTSKLFNEE RLIDKTKVTY LKWLPESESL FLASHASGHL YLYNVSHPCT STPPQYSLLK 240
241  QGEGFAVYAA KSKAPRNPLA KWAVGEGPLN EFAFSPDGRH LACVSQDGCL RVFHFDSMLL 300
301  RGLMKSYFGG LLCVCWSPDG RYVVTGGEDD LVTVWSFTEG RVVARGHGHK SWVNAVAFDP 360
361  YTTRAEEAAS ASADGDPSGE EEEPEVTSSD TGAPVSPLPK AGSITYRFGS AGQDTQFCLW 420
421  DLTEDVLSPH PSLARTRTLP GTPGATPPAS GSSRAGETGA GPLPRSLSRS NSLPHPAGGG 480
481  KAGGPNASME PGIPFSIGRF ATLTLQERRD RGAEKEHKRY HSLGNISRGG SGGNSSNDKL 540
541  SGPAPRSRLD PAKVLGTALC PRIHEVPLLE PLVCKKIAQE RLTVLLFLED CIITACQEGL 600
601  ICTWARPGKA FTDEETEAQA GQASWPRSPS KSVVEGISSQ PGSSPSGTVV           650
```

**Fig. 4.** Amino acid sequence of mouseDMWD with all possible trypsin cleavage sites.
Amino acids in bold are potential trypsin cleavage sites. Underlined are the a-c strand areas of the four putative WD-repeats. Also underlined is the region of the fifth area with homology to WD-repeats. In italics, amino acids are given that were found to be very conserved in the blockmaster assay.

Together, this indicated that proteolytic attack mainly occurred in the N- or C-terminal segments but not in the mid-portion of the protein. At very high trypsin concentrations (lane10) the DMWDfl protein was cleaved at multiple locations and fragments produced had lost their antigenic properties resulting in a complete loss of signal with antibody abB13. Protein samples that were placed at 37°C, without trypsin, did not show any noticeable proteolytic breakdown, other than the faster migrating protein product in lane 1.

To obtain a better picture of the sites at which DMWD was most vulnerable to trypsin cleavage, we compared the tryptic digestion patterns of pure DMWDfl with those of DMWD segments produced as chimeric GFP-fusion polypeptides. Segments analyzed comprised either the core with the four WD-repeats and the C-terminus or the N-terminal area.

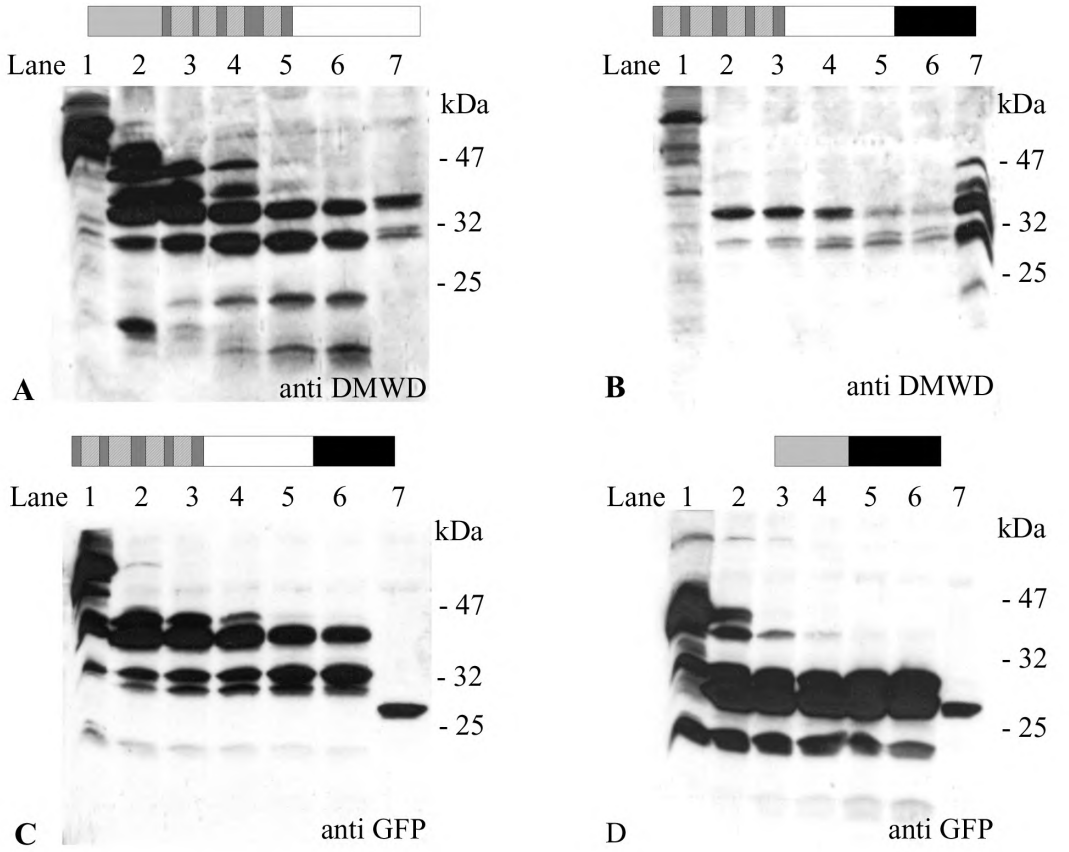



**Fig. 5.** Western blots of DMWD protein products.
Panel A: Lane 1 - 6 DMWDfl (protein product of pSG8-DMWD$_{fl}$) digested with trypsin (final concentration 4.2 μM) for 0, 2.5, 5, 10, 30 and 60 min. respectively Lane 7 WD-repeat polypeptide (protein product of pSG8-DMWD$_{129-650}$-EGFP) digested with trypsin (final concentration 4.2 μM) for 10 min. Panel B: Lane 1 - 6 WD-repeat polypeptide (protein product of pSG8-DMWD$_{129-650}$-EGFP) digested with trypsin (final concentration 4.2 μM) for 0, 2.5, 5, 10, 30 and 60 min. respectively. Lane 7 DMWDfl (protein product of pSG8-DMWD$_{fl}$) digested with trypsin (final concentration 4.2 μM) for 10 min. Panel C: WD-repeat polypeptide (protein product of pSG8-DMWD$_{129-650}$-EGFP) digested with trypsin (final concentration 4.2 μM) for 0, 2.5, 5, 10, 30 and 60 min. respectively. Lane 7 shows the non-digested EGFP protein. Panel D: Lane 1 – 6 DMWD N-terminal polypeptide (protein product of pSG8-DMWD$_{1-135}$ –EGFP) digested with trypsin (final concentration 4.2 μM) for 0, 2.5, 5, 10, 30 and 60 min. respectively, all detected with αGFP. Lane 7 shows the non-digested EGFP protein. Proteins in panel A en B were detected with anti-DMWD abB13, proteins panel in C en D were detected with αGFP.

In Fig. 5 Western-blot patterns are shown of the different fragments formed after incubation with trypsin (standard concentration 4.2 uM) for periods between 0 and 60 minutes in complete COS-1 cell lysates. Panel A shows DMWDfl digested with trypsin for 0 to 60 min (lane 1 –6). As a control the WD-repeat polypeptide segment (pSG8-DMWD$_{129-650}$-EGFP) was also cleaved with trypsin (lane 7; 10 min incubation). Clearly, the WD-repeat segment and the DMWDfl protein yielded similar remaining bands (one of 40 kDa and a doublet of 30 kDa) of the DMWD protein after trypsin digestion (lane 6 and 7). This implicated that the most resistant fragment was located in the overlapping area of these two proteins.

In Panel B the analysis of the vulnerability of the WD-repeat polypeptide towards prolonged trypsin digestion is shown (lane 1-6). As a control the DMWD$_{fl}$ protein (pSG8-DMWD$_{fl}$) was also cleaved with trypsin (lane 7; 10 min incubation). Detection by polyvalent DMWD antiserum revealed that the three most resistant bands ran with the same apparent molecular weights as the trypsin-resistant fragments from DMWD$_{fl}$ protein (compare lanes 6 and 7 panel A and lanes 6 and 7 panel B). The product of approximately 40 kDa yielded the most intense signal at relative short trypsin-incubation periods, whereas the products of approximately 30 kDa appeared more intense after prolonged trypsin treatment. To us this suggested that there was some sort of precursor-product relationship between the 40-kDa and 30-kDa products. In panel C a Western-blot analysis of tryptic digests (lane 1 – 6) of the GFP-tagged WD-repeat polypeptide (pSG8-DMWD$_{129-650}$-EGFP) is shown. This analysis revealed cleavage products with apparent molecular weights of approximately 32 and 45 kDa for the C-terminal segment spanning the WD-repeat. As a control undigested pure EGFP product, migrating at an apparent MW of 27 kDa, was included (panel C, lane 7).
When this COS-1 cell produced EGFP was incubated with trypsin it appeared totally resistant to treatment (for periods up to 60 min; results not shown). As polyvalent GFP-specific antiserum was used for detection this meant that the two protein products seen represent the GFP (27 kDa) moiety plus fused proteolytic remnants of the DMWD protein. If we assume that the C-terminus of the DMWD protein in the fusion construct still adapts its normal folding characteristics and was not protected by steric hindrance by the adjacent GFP structure, our findings can only be explained by assuming that susceptible tryptic sites are located at approximately 130 to 170 amino acids and approximately 80 amino acids upstream of the C-terminus but that other sites in this area are relatively resistant to trypsin.
Panel D shows a similar analysis for the N-terminal portion of the DMWD protein. Here it was evident that this region of DMWD was highly vulnerable and immediately cleaved, after already 5 min the resulting protein product had almost the same apparent MW as the GFP proper (lane 3).

## Discussion

*WD-repeats in DMWD*

WD-repeat proteins consist of minimally four WD-repeats. Four WD-repeats is also the minimal number of elements required to form a rigid β propeller ring structure. In the initial computer analysis of mDMWD and its homologue in *A.nindulans* CreC, five putative WD-repeat-like areas were identified. Here we demonstrate that the length of the segment in between the fourth and the fifth WD-repeat segments was 8-fold (mDMWD) and 5-fold (CreC) longer, respectively, than the average segment connecting adjacent repeat elements elsewhere in the protein. Typically, WD-repeats within proteins are conserved not only with respect to the type of side chain but also in their inter-WD-repeat spacing [158,199,222].

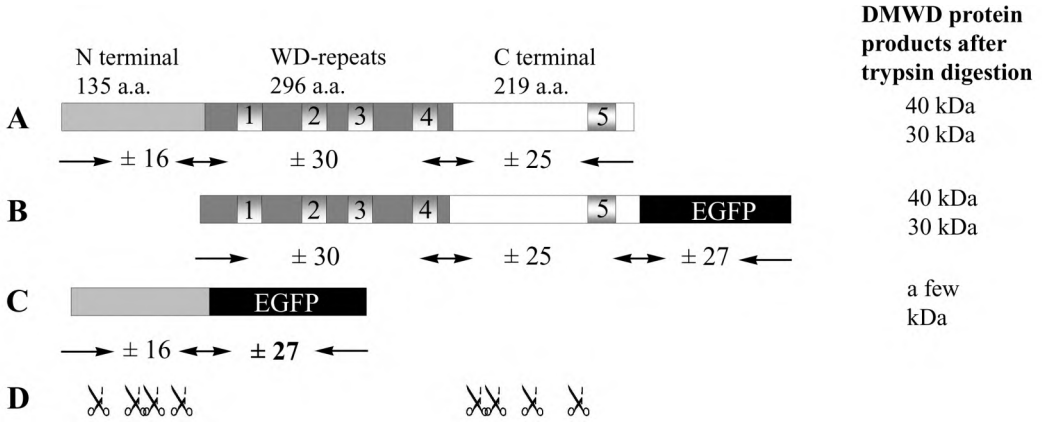
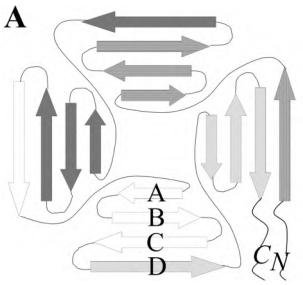



**Fig. 6.** Partial trypsin digestion map of the DMWD protein.
Schematic drawings of different structural elements in the full-length protein (panel A), the WD-repeat rich core and C-terminus as an EGFP-fusion protein (panel B), the N-terminal region of DMWD as a fusion protein with EGFP (panel C) are given. A map is given (panel D) with the location of preferential trypsin cleavage sites. Clustering of trypsin sites occurs throughout the N-terminus and in the middle of the C-terminal area. Note that no trypsin cleavage was observed in the WD-repeat areas (1-4) and also not in the region where the segment with WD-repeat homology (5) is located

We found (not shown) that inter WD-repeat distances in other WD-proteins vary by not more than a factor of two from the average length, with very few exceptions. Based on these observations and theoretical considerations we propose that DMWD in mouse and CreC in Aspergilus nidulans are both four-WD-repeat proteins. Most likely, the fifth region with homology to WD-repeats does not form an integral part of the WD-repeat core structure.

*Biochemical evidence for a propeller ring structure in DMWD.*
To obtain further independent support for presence of a propeller ring, we probed the structure of the mDMWD protein with a biochemical assay based on partial tryptic cleavage. Other groups have already reported that WD-repeats when folded into the propeller-like structures are relatively immune to trypsin digestion [62]. In Fig. 6 the interpretation of our trypsin digestion experiments are summarized (compare also Figs. 4 and 5). We observed that the N-terminal site of DMWDfl was highly susceptible to cleavage by trypsin. In the C-terminal region, we observed clustering of susceptible sites adjacent to regions with relative low trypsin sensitivity (see Fig. 4). Strikingly, the core region of the protein with the four adjacent WD-repeats was almost entirely inert against proteolytic cleavage. Another area with low sensitivity to trypsin attack overlapped with the fifth putative WD-repeat in the C-terminal part. Only if this latter WD-repeat forms an integral part of one large and rigid propeller-ring structure then we could have expected to see full protection of the entire region (repeats 1-5). As this was not the case (See Figs. 4 and 5) our results support a model for the DMWD structure with a core of four WD-repeats.

Based on the sequence analogy to the WD-repeat region in the G β subunit of heteromeric G-proteins, and the three-dimensional crystal structure thereof, we propose in Fig. 7 a 2-D working model for the folding of the WD-repeat core of mDMWD. The propeller should consist of four blades, which each contain three β strands of one repeat and one β strand of the next repeat (see Fig. 7). The last blade is composed of strands A – C of WD4 and strand D of repeat WD1. The N-terminal part and the C terminal part will presumably not fold together with the repeats and will stick out.




**B**

| | Strand D | Strand A | Strand B | Strand C |
|---|---|---|---|---|
| | -----▶ | -----▶ | -----▶ | -----▶ |
| **WD1** | DTSKLFNEERL | IDKTKV TYLKWL | PESES LFLASH | ASG HLYLYN |
| **WD2** | VSHPCTSTP-A | VGEGPL NEFAFS | PDGR HLACVS | QDG CLRVFH |
| **WD3** | FDSMLLRGLMK | SYFGGL LCVCWS | PDGR YVVTGG | EDD LVTVWS |
| **WD4** | FTEGRVVARGH | GHKSWV NAVAFD | PYT-Y RFGSAG | QDT QFCLWD |

**Fig. 7.** WD-repeats in mDMWD.
Panel A: Hypothetical 2D drawing of the folding arrangement of the four WD-repeats in mDMWD. Each blade consists of 3 β strands of one repeat (strand A – C) plus one β strand of the next repeat (strand D). All β strands of one repeat element have the same colour/pattern.
Panel B: Sequence alignments of strands A-D in the four WD-repeats in mDMWD. Note that strand D of WD2 and the A-B loop of WD4 are not shown totally (-), for better interpretation.

*Structural arrangement of other conserved areas in DMWD*

Only few WD-repeat proteins solely consist of a WD-core, without any N-terminal or C-terminal extensions or other structural or functional domains. Examples are the SEC13 protein, which is active in vesicular traffic [62] or the RACK1 protein, which is involved in signal transduction [184]. Most WD proteins do have N and/or C terminal extensions and in some of them the WD-repeat area is connected to other motifs like the F-Box, the HMG-box or the SOCS box. The F-box is a 40 amino acid motif that was first found in cyclin F [10]. WD-repeat proteins with an F-box (named Fbws [28]) mainly act as regulators like Cdc4p that controls cell division by degrading G1 cyclins and cyclin dependent kinase inhibitors [41] or Met30p, which represses methionine biosynthetic gene expression when availability of L-methionine is high [200,212]. So even though Fbws have both motifs they still bind to different protein partners.

The HMG-box is an 80 amino acid motif with a flattened triangular fold molecular structure that recognizes and binds architectural features in DNA [229]. Proteins that have both the HMG-box and WD-repeats combine direct DNA binding (HMG-box) with protein-protein interactions (WD-repeat). AND-1 (acidic nucleoplasmic DNA-binding protein) is such a protein; it has a single HMG-box and seven WD-repeats. AND-1 binds DNA and the HMG-box specifically binds to four-way DNA junctions, possible protein partners that could bind to the WD-repeats are not elucidated yet [115]. The third known box that was found in combination with the WD-repeat is the SOCS-box. This motif was first identified in the suppressor of cytokines signalling family and is found conjunct to several other motifs in proteins: SH2 domains in SOCS proteins, WD-repeats in WSB proteins and SPRY domains in SSB proteins [88]. The function of this 50 amino acid C-terminal domain has not been elucidated yet it might however function in a direct inhibition of Jak or receptor tyrosine kinases [107].

We checked for DMWD if the N and /or the C terminal part of the protein had any homology with known protein motifs. The N-terminal part of mouseDMWD (amino acid positions 31 to 76) showed the strongest homology to synapsin proteins. These proteins have a proline rich sequence, which can possibly bind to SH3 domains [164] and have a role in signalling cascades. Proline rich sequences were also found in many transcription factors, and are involved in protein-protein interactions, in some instances mediating transcriptional activation [111,149,233].

Strikingly, the proline-rich sequence in mDMWD (N-terminal part consists of 22 proline residues in a stretch of 100 amino acids) is also found in the N-terminal part of CreC [217] but not in any of the other presumed DMWD orthologues in human, *Drosophila, S. pombe* or *A.thaliana*. Also the more extensive synapsin motif is lacking in these proteins, which makes it highly improbable that is serves as a function motif.
Furthermore, only weak homology to two other motifs was identified in the total mDMWD sequence, the Tub family motif and the Rhodopsin-like GPCR superfamily signature. The probability scores of the Rhodopsin-like GPCR superfamily signature were low and therefore we consider it unlikely that this motif as a functional role in DMWD proteins.
The Tub motif had higher probability scores. This motif was discovered in Tub and Tub-like proteins and these proteins play an important role in maintenance and function of neuronal cells during development and post-differentiation [95]. Also based on the spatial and temporal expression pattern of mDMWD in brain (this thesis, chapter 2), this motif could have functional significance, but further study is necessary to investigate this point.
The C-terminal area of DMWD has a homology to WD-repeats but was shown in this study not to fold together with the other 4 WD-repeats and therefore not considered as a true WD-repeat. It is likely, however that this conserved area has a biological (conserved) function. Todd [217] and co-workers showed in their studies that this area in CreC is required for catabolite repression. Whether it could serve a similar role in DMWD remains to be resolved.

*Possible protein partners for DMWD*
Because of the way WD-repeats fold, they are thought to bind to very different proteins.
Their different binding areas (top, bottom and side of the propeller) make them very suitable for these functions and it could explain why they do not have an overall common function related to the WD-repeat. Some of the biological functions of WD-repeat proteins are: a role in RNA processing (PRP17), vesicular traffic (SEC13) [62], signal transduction (G-proteins) [204], cytoskeleton assembly (coronin) [40,161] and cell division (CDC20) [129,130]. This list gets more extended every month as new biological functions are assigned to WD proteins.

Recently, it was shown that the CreC protein (the DMWD *A.nidulans* homologue) interacts with CreB under catabolite repressing and derepressing conditions and in doing so stabilizes or activates the CreB enzyme. CreB is a deubiquinating enzyme and has a human homologue called ubiquitin hydrolysing enzyme 1 (UBHI). CreC has four PEST sequences, these are hydrophilic stretches of polypeptide sequences that are rich in proline, glutamic acid, serine and threonine, flanked by positively charged residues, and implicated as signals for proteolysis [134,178,183]. The presence of PEST sequences is correlated with ubiquination and rapid degradation [178]. Only the PEST sequences that are exposed on the surface of a protein lead to degradation [203]. CreC most likely stabilizes the CreB enzyme by masking the PEST sequences. CreB in its turn stabilizes yet another protein, CreA, by removing covalently bound ubiquitin moieties. CreA is a domain DNA-binding regulatory protein involved in carbon catabolite repression in *A. nidulans* [134]. If we extrapolate these findings to mDMWD, the DMWD protein would, through this interaction, be involved in a proteolitic/(gene)-regulation pathway.

*CreC and other DMWD homologous proteins*
The CreC protein and all the putative homologous DMWD proteins were classified with the WD-repeat prediction computer program, as a 4-WD-repeat protein, although for some five or more putative WD-repeat areas were found in the original search. Although we did not do any biochemical experiments with the CreC protein or any of the other homologous proteins, we would like to propose that based on the results obtained for the DMWD protein, the DMWD homologous proteins are 4-WD-repeat proteins, as well as DMWD.

*Overall conclusion*

We provided here bio-computational and biochemical evidence that the name DMWD has been rightfully assigned, and that the mouse DMWD proteins may indeed be considered a genuine member of the WD-protein family. Moreover, biochemical evidence showed that the repeat-segment in the mouse DMWD proteins is likely to be folded into a β propeller like structure. If CreC were a functional orthologue of mDMWD it would be likely for DMWD to be involved in a regulatory (protein degradation) pathway.

# Chapter 6

## The Myotonic Dystrophy WD protein (DMWD) homologue in C. elegans has a role in germ line development and embryogenesis

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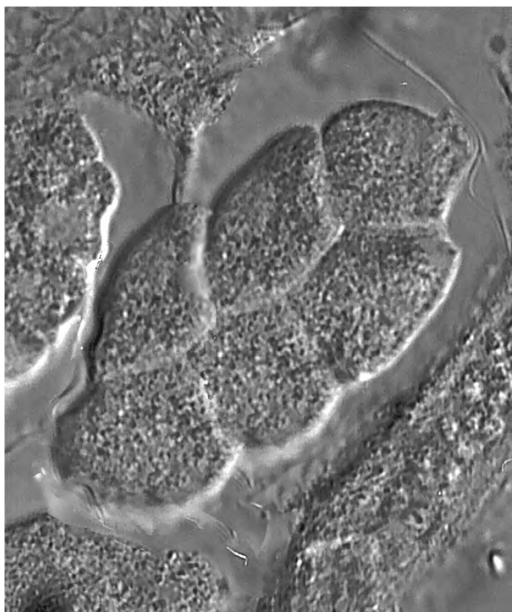

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**Summary**

The nematode *C. elegans* has become the model organism of choice to analyze gene function, not only because its genome sequence and entire body plan is known, but also because many dedicated reverse genetic techniques are available to study gene product significance. Here we have used *C. elegans* to examine the normal biological role of the ceDMWD, the structural and presumed functional orthologue of the human DMWD gene. Human DMWD is a candidate for being involved in the complex clinical manifestation of Myotonic Dystrophy (DM) or Steinert disease. In this chapter we report on the study of ceDMWD RNA and protein localization, and the effects of use of RNA interference (RNAi) to disrupt gene function. We find that ceDMWD RNA and protein are expressed in embryos and in the gonads of adult hermaphrodites and we also find that the RNAi leads to gonadal defects in gravid F1 adults and embryonic death in part of the F1 offspring. Based on these observations we propose that ceDMWD has a role in germ line development and embryogenesis of *C. elegans.*

## Introduction

*C. elegans* is a nematode that is used in many laboratories to study animal development, basal aspects of behaviour and gene function. The organism has gained enormous popularity because of its short life cycle (3-days), small size (1.2 mm long adult) and easy maintenance. *C. elegans* is a hermaphrodite and can produce 200 to 300 embryos over the course of several days [182]. Because many labs chose to work with *C. elegans* during the last few decades there is an enormous amount of information about cell fate during development. As a consequence, a complete description of every somatic cell lineage is available, starting at the one cell embryo stage throughout development until the generation of the adult. Moreover, its genome has now been completely sequenced [1] and many techniques have been developed to analyze gene product function in *C. elegans*.
For all these reasons this nematode was our model system of choice for a systematic study of the normal biological role of the ceDMWD gene, the orthologue of the Myotonic Dystrophy WD-repeat gene (DMWD) of humans (also designated DM-59) and mice (DMR-N9). DMWD was first identified when the mutation underlying the multisystemic disorder Myotonic Dystrophy was identified and the complete human chromosome 19q 13.2-3 segment carrying the DM locus was charted [99]. Even with the nature of this mutation revealed, an expansion of a $(CTG)_n$-repeat in the 3'-UTR of the Myotonic Dystrophy Protein Kinase gene (DMPK), it remained difficult to explain the diverse symptoms seen in DM. Almost certainly the complex clinical manifestation of DM cannot be caused solely by quantitative effects on the expression or qualitative effects on the serine/threonine-specific kinase activity of DMPK, simply because various tissues are involved where the DMPK gene is not expressed. In human and mouse another gene, DMWD, is situated immediately upstream of the DMPK gene, whose transcription unit may be contiguous with that of DMPK [100,138,195]. Our group therefore decided to the study of consequences that the (CTG)-repeat increase might have on DMWD activity. DMWD gene products in mice (in this chapter referred to as mDMWD) are mainly expressed in brain and testis, but also at low level in many other tissues. In brain it is located at sites that contain many synapses and in testis it is found in Sertoli cells and sex vesicles of pachytene spermatocytes (this thesis). These locations, however, did not give us reliable clues on the possible function of the DMWD gene.
Sequence analysis revealed that mouseDMWD (and human DMWD) has a proline rich N-terminal region, four complete WD-repeats and a C-terminal area with weak WD-repeat consensus homology. This C-terminal region may have another function different than the other WD-repeats (see chapter 5, this thesis). WD-repeats are thought to be involved in protein-protein interaction but their exact nature of these engagements is not known. Earlier we demonstrated that the predicted ceDMWD protein is 100 amino acids longer than the mammalian protein but otherwise has all characteristic domain features of the mDMWD protein. Here we report on protein and RNA localization and RNA interference (RNAi) studies in *C. elegans* that demonstrate that DMWD protein and mRNA occur in early embryos and adult gonads. This expression distribution is somewhat reminiscent to that in mouse where DMWD is also prominently expressed early in development and in (adult) testis. When RNA interference was used to analyze the effects of ceDMWD loss-of-function, we found that the progeny of injected worms had gonadal defects and part of the offspring of the F1 generation arrested, mostly before mid-embryogenesis. These findings led us to propose that ceDMWD is involved in embryogenesis and germ line development.

## Materials and methods

*Worm culturing*
*C. elegans* (N2 *var.* Bristol) were maintained under standard conditions [22].

*Bio informatics*
Global alignments of putative DMWD proteins were done by using software available at the Home Page of the Swiss Institute for Bio informatics at www.ch.EMBnet/software/lalign, using scoring matrix Blosum62, gap penalties of -10/-5. Sequences used were mouseDMWD (SwissProt accession no. Q08274), human DMWD (SwissProt accession no. Q09019), *C. elegans* C08B6.7 (Genbank accession no. Z72502), *Aspergillus nidulans* CreC (Genbank accession no.AF136452), *Schizosaccharomyces pombe* YDE3 (SwissProt accession no. Q10437), *Arabidopsis thaliana* F4P12.90 Genbank accession no. T45879), and CG6420 gene product *Drosophila melanogaster* (gi 6729490).

*Start site and SL1 splicing*
To analyze the translational start site of ceDMWD we checked for SL1 (Spliced Leader 1) and SL2 (Spliced Leader 2), as most *C. elegans* mRNAs contain a *trans-spliced* SL1 or SL2 leader sequence. This was done with the Titan One Tube RT-PCR kit (Titan, Roche Diagnostics) according to the manufacturer's instructions. The primers SL1: 5´-GGTTTTAATTACCCAAGTTTGAG-3´, SL2: 5'-GGTTTTAACCCAGTTACTCAAG-3' and JWCE7.rev: 5´-CGGGATCCAAGCTTACACGAACAGTAGAGCCC-3´ were used with mRNA collected from gravid adults (kindly provided by Dr. T. Fukushige, University of Calgary).

*Northern analysis*
To analyze the mRNA expression in the different developmental stages, we isolated total RNA of embryos, L1, L2, L3, L4, young adult hermaphrodites (containing no embryos, analyzed by microscope), and gravid hermaphrodites (containing embryos). Adult worms were grown and treated with alkaline hypochloride solution to isolate the embryos. A portion of these embryos were used for RNA and protein isolation, the rest were used to start the worm culture. All remaining stages were monitored by microscopy, and worms were kept at 20°C during culture. Worms of each stage were washed 3 times with PBS and put in an appropriate volume of RNAzol™B (50 mg/ml of RNAzol, Campro Scientific). After thorough homogenization of the worm-RNAzol mixture, 1/10 volume of chloroform was added, mixed and centrifuged at 13.000 rpm for 20 min. at 4°C. RNA in the supernatant was precipitated on ice in a new tube with one volume of isopropanol. The mixture was centrifuged as above, and the pellet was washed twice with 75% ethanol, dried and kept under 70% ethanol in NSE (50 mM NaAc, 0.2% SDS, 2 mM EDTA) at –20°C. RNA concentrations were determined from $E_{260\ nm}$/ $E_{280\ nm}$ readings and comparison of intensities of staining of rRNAs on an analytical 1% agarose test gel.
Northern blot analysis was carried out using 10 µg of total RNA by o/n electrophoresis on a 1% agarose/formaldehyde/MOPS gel. After the gel was photographed, the RNA was transferred to a nitrocellulose membrane (Schleicher and Schuell) and cross-linked. Hybridization of the membrane with a random labelled ($\alpha$-$^{32}$P-CTP, Amersham, England) probe (± 2 kb EcoRI-XhoI fragment) was done o/n at 65°C. The probe contained the cDNA yk48 (kindly provided by Y. Kohara, Gene Network Lab, Japan), which corresponds to nucleotides 313-2213 of ceDMWD plus 273 nt of the 3'-UTR. Radioactive signals on the membrane were visualized by exposure to Kodak X-OMAT film (18 to 72 hours).

*In situ hybridization*

Whole-mount *in situ* hybridization for the detection of RNA in embryos was done as described by [192]. In short, embryos or adults were placed onto microscope slides (that have 3 square 14x14mm wells surrounded by a thin hydrophobic coating similar in thickness to a *C. elegans* embryo) coated with polylysine, permeabilized by freezing and fixed with methanol and formaldehyde. Embryos and adult worms were then incubated o/n with a single stranded digoxigenin-labelled ceDMWD DNA probe. For these experiments pBlueSKyk48: pBlue with the ± 2 kb EcoRI-XhoI fragment described above was used as the probe. The T7 primer was used for the generation of anti-sense probe and the T3 primer for the sense control probe. Both probes were used in a 50ng/well concentration with 35 µl/well. After incubation slides were washed extensively to remove excess probe, and the slides were incubated with alkalinephosphatase (AP)-anti-DIG conjugated antibody (1:2500 in PBST). The AP-conjugated antibody was visualized by NBT/X-phosphate (4-Nitro blue tetrazolium chloride/5-Bromo-4-chloro-3-indolyl-phosphate; Boehringer Mannheim) with the addition of DAPI (4′, 6-diamidine-28-phenylindole dihydrochloride; 1µg/ml) to each well. The colour reaction was stopped after 30 min to 1 h. by washing in PBS, and the worms/embryos were mounted in a 70% glycerol solution and analyzed under the microscope.

*Antibody staining*

To produce a polyclonal antibody against ceDMWD two rabbits (255 and 256) were injected three times each with 400 µg of the purified peptide KKVSHDRLTVLEFREDC coupled to KLH (commercial Ab production procedure by Eurogentec, Belgium). After the first injection, the serum of both rabbits was tested for reactivity to the peptide. Ten weeks later, after two additional injections, the serum was collected and checked again for reactivity. Both antisera showed reactivity, and a stock was made of the serum ($2^{nd}$ bleed) and kept at – 20°C or – 80°C. The mixed serum was purified from *E. coli* proteins by use of the Immobilized *E. coli* Lysate kit (Pierce, Rockford, Illinois, USA) according to manufacturer's instructions. In each experiment this mixed serum was used (1:25 to 1:50) together with DAPI to stain DNA and with monoclonal anti-tubulin (1:50) [171] to check for permeability.

Antibody staining of embryos and gonads were performed by using the methanol-acetone method of Albertson with modifications of Kemphues or the sandwich method of Siddiqui [3,183,196]. Briefly, adult worms were collected from plates and placed on microscope slides coated with polylysine, covered with coverslips and quickly frozen on dry ice. After a minimum of 30 min the coverslips were snapped off, and the microscope slides were incubated in –20°C methanol for 15 min, transferred to –20°C acetone for 2 min and incubated o/n in PBST at 4°C. The next day the worms were pre-incubated for 30 min with 5% normal goat serum (NGS) in PBST followed by incubation with the appropriate antibody for at least 2 h in a humid chamber at room temperature. Preparations were washed with PBST and incubated with the secondary antibody (1:100 in PBS, FITC-conjugated or Cy3-conjugated goat anti-rabbit and TRITC- or Cy2-conjugated donkey anti-mouse, Jackson Immuno research, USA). Stained embryos and gonads were immersed in DAPI for maximal 10 min prior to mounting in a 1mg/ml solution of p-phenylenediamine in 90% glycerol to reduce fluorescence fading. Photographs were taken with a Zeiss Axioplan fluorescence microscope with Techpan film (Kodak) developed at ASA100.

For application of the sandwich methods (optimized by T. Fukushige, University of Calgary) worms were grown on 10 X peptone plates and washed of with M9 buffer. These worms were filtered through a 40-50 µM nylon mesh to remove larvae. The adult worms were resuspended in M9 buffer and washed three times, and then they were fixed in 5% paraformaldehyde (PFA) in 0.25M phosphate buffer on ice for 5 min.

After this the worms were centrifuged and mounted on 0.2% gelatine coated slides, covered by a coated-coated slide, squashed and frozen on dry ice for at least 30 min. The sandwiched slides were snapped open by a razor blade and each slide was incubated for 6 min each in –20°C acetone/methanol (1:1), -20°C 90% ethanol, ice-cold 70% ethanol, ice-cold 50% ethanol, ice-cold 30% ethanol, room temperature PBS and o/n at 4°C in PBST (0.1% Triton). Antibody staining was performed as described above.

*RNAi*

RNAi was performed as previously described by Fire et al [51]. Briefly, a 2.2 kb BamHI cDNA fragment, containing part of the cDNA yk48 was cloned into the BamHI site of the pBlueSK. This vector was used to amplify the cDNA fragment by PCR with M13 forward and reverse primers. This DNA contained both the T3 and T7 promoter region, which were used, according to the manufacturer's instructions with the Ambion system RNA-polymerase (Ambion, Austin, TX), to produce single stranded RNA. The ssRNA was precipitated and resuspended in RNase free TE. Equal amounts of T3 or T7 generated ssRNAs were mixed, heated at 65°C for 10 min and placed at 37°C for 1 h. Single and double stranded RNA was checked on a standard agarose gel, and the dsRNA was stored at –20°C until further use, for up to a week. For each RNAi experiment 2.5 µg/µl was used to inject wild-type worms. Injected worms were allowed to recover in M9 buffer for 5 to 10 min and placed at 15°C o/n on normal plates. The next morning each injected hermaphrodite was placed on a separate plate and incubated at 20°C to produce progeny, the next day the mothers were transferred to new plates at 20° or 25°C. The laid embryos were kept at 20° or 25°C to check for hatching. Since in these experiments most progeny of injected mothers hatched, we placed 5 to 10 of these progeny (at stage L3/L4) from each injected hermaphrodite individually onto plates and let them lay eggs at 20°C. Every day these worms were individually transferred to new plates and the F2 progeny (at 20°) was checked for hatching. As a control in these experiments, wild type worms were injected with RNAse free TE.

*Embryonic phenotypes*

Progeny of RNAi injected worms were scored for embryonic lethality among their progeny. To this end L4 worms were transferred to individual plates and allowed to produce eggs o/n at 20°C. The next day worms were transferred again to new plates and 24 h later, the embryos on the first plate were checked for hatching. Embryos were collected from worms that showed high incidence of embryonic death in their earlier broods, by scissoring the gravid adults in the middle with two 21-gauge needles in egg-buffer [46]. Two or four-cell embryos were transferred to an agarose pad, covered with a coverslip and sealed with Vaseline to prevent dehydration. Embryos were monitored for 12 to 20 h at 20°C, images of the embryos were recorded on tape every 6 or 12 seconds, using Nomarski optics and an Zeiss Axioplan microscope.

## Results

*Homology to mammalian DMWDs*

To identify possible orthologue(s) of DMWD in *C. elegans,* we performed a BLASTP search with mDMWD of the *C. elegans* wormpep database (Sanger Institute at BCM search launcher, http://www.searchlauncher.bcm.tmc.edu/seq-search/protein-search-genomes.html). After the *C. elegans* genome project was completed, in 1998, we detected only one gene C08B6.7 in the *C. elegans* database that showed high similarity (high score of 729 and probability of $2.2 \times e^{-76}$) to mDMWD.

This protein (designated ceDMWD in this chapter) showed 33.1% identity and 58.8% similarity to mDMWD (see Fig. 1A and Table. 1). CeDMWD is homologous to mDMWD over almost the entire length of the protein (see Fig. 1B) but shows the highest homology in the WD-repeat region (68% similarity). The C-terminal region of mDMWD (amino acid 550 to 610) also seems to be well conserved between mouse and *C. elegans* but as demonstrated in chapter 5 (this thesis), this region in DMWD is presumably not engaged in functional interaction with the other four WD-repeats. In chapter 5 we only searched the six proposed orthologues (human DMWD, *C. elegans* C08B6.7, *Aspergillus nidulans* CreC, *Schizosaccharomyces pombe* Yde3, *Arabidopsis thaliana* F4P12.90, T45879 and *Drosophila melangonaster* AAF56618.1) for the most conserved segments with the Block Master programme. Here we analyzed the identities and similarities across the full-length of all putative DMWD homologues (Table 1).

**Table 1.** Percentages of amino acid similarity (in italics) and identity (in bold) in alignments of seven DMWD proteins.

| | Human DMWD | Mouse DMWD | D.melano-gaster | C. elegans | S. pombe | A. nidulans | A. thaliana |
|---|---|---|---|---|---|---|---|
| Human | - | 80.0 | 40.1 | 51.8 | 43.3 | 45.7 | 45.6 |
| Mouse | 77.2 | - | 48.1 | 58.8 | 43.6 | 47.4 | 51.0 |
| D.melanogaster | 30.2 | 34.9 | - | 55.4 | 38.9 | 44.2 | 48.2 |
| C. elegans | 29.7 | 33.1 | 27.6 | - | 42.3 | 44.3 | 48.9 |
| S. pombe | 13.5 | 23.1 | 19.4 | 20.4 | - | 60.2 | 48.5 |
| A. nidulans | 17.3 | 18.3 | 21.3 | 17.4 | 32.4 | - | 52.8 |
| A. thaliana | 16.3 | 29.2 | 21.2 | 25.2 | 24.7 | 28.2 | - |

Human (Swiss prot: Q09019), mouse (Swiss prot: Q 08274), *D. melanogaster* (gi: 7301495), *C. elegans* (C08B6.7), *S. pombe* (Yde3 gi:1723547), *A. nidulans* (CreC gi: 7528185) and *A. thaliana* (gi 6729490).

All proteins show high similarity to the mouse DMWD protein with identity ranging between 18.3% - 77.2% and similarity between 43.6% - 80.0%. The lowest identity score (13.5%) was between the DMWD proteins of human and *S. pombe*. At first sight this might not appear significant for a true orthologue, but when compared for similarity, the score is at 43.3% between these two species.
As *C. elegans* only had one DMWD-like protein and was considered the most easily accessible model organism to study, we went to analyze the different aspects of ceDMWD gene expression, starting out with the RNA *trans*-splicing.

*RNA splicing*
In *C. elegans* many primary RNA transcripts are *trans*-spliced. Various mature mRNAs therefore contain an identical short leader sequence (the spliced leader, SL) at their 5'-end. The most commonly used splice leaders in *C. elegans* are SL1 and SL2, both 22-nucleotides long. *Trans*-splicing is functionally closely related to *cis*-splicing (intron-removal) in *C. elegans* [20].

**A** Identity: 33.1%, Similarity: 58.8%

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              10        20        30        40        50        60        70        80
ceDMWD MHQHMLGPSTSLGGTNNSALQSSVLSEGVLLSHSQPTREEIKKNFETREGVYRSVPSAEFSRPRPLPQYHMPPGIASAAA
       :     :  ... .  ..       :  :    .: ::       . .:        .:  . ..: : :      :: :. :.
mDMWD  M-----GDCAEIKSQFRTREGFYKLLPGDATRRSGPT------SAQT------PAPP-QPTQPPPGPAAASGPGAAGPAS
                  10        20        30                  40         50         60
                90        100       110       120       130       140       150
ceDMWD SQIAAA-GSTVRVSFLNGAEKSVESPEAVAPTSSTYEHHKNEPNGARIDMVDEAQADKICFNVGKELYVFS--YR-GTQT
       :   :. :    .  .. .   . .:....   ::          :.:        : .:..:::.:.::: .    : :.
mDMWD  SPPPAGPGPGPALPAVRLSLVRLGDPDGAGEPPST-------PSGLG------AGGDRVCFNLGRELYFYPGCCRSGSQR
             70        80        90              100               110       120       130
        160       170       180       190       200       210       220       230
ceDMWD ETDLSRPIDKRVYKGTSPTYHAFNQESAKNGSCQLLIGFTLGQLQIIDPLEKSSSSPFSRLYNEDRYIEKTSVTCIRWLP
       . ::..:::::.::::.:: : ::: .: . . .:::.::. ::.: .: ..:..:    .:.:::.: :.::.:: ..:::
mDMWD  SIDLNKPIDKRIYKGTQPTCHDFNQFTAATETISLLVGFSAGQVQYLDLIKKDTS----KLFNEERLIDKTKVTYLKWLP
              140       150       160       170       180           190       200
        240       250       260       270       280       290       300       310
ceDMWD GDSNIFLASYVSGNLYVYDQRISAASSNNNGSSQPPPWTIHKEGDKFAIHTWKGKVQRNPVTRWQIGEGSIHQFSFSGSD
       .....::::..::.::.:.  .:   .     : :: ... :.:. :::.. :.:. :::...: .::: ...:.::  :
mDMWD  ESESLFLASHASGHLYLYN--VSHPCT-----STPPQYSLLKQGEGFAVYAAKSKAPRNPLAKWAVGEGPLNEFAFS-PD
         210       220         230             240       250       260       270
        320       330       340       350       360       370       380       390
ceDMWD GKMMATVSHDGFLRIFNYHAQELLAVMKSYFGGLLTLSWSPDAKLIATGGEDDLLTVYSVAEKRVVCRGQAHKSWVSQVK
       :. .::::.:: ::.:.. .. : ..::::::::: . :: ::.. ..::::::::.::.: .: :::.::..::::::. :
mDMWD  GRHLACVSQDGCLRVFHFDSMLLRGLMKSYFGGLLCVCWSPDGRYVVTGGEDDLVTVWSFTEGRVVARGHGHKSWVNAVA
       280       290       300       310       320       330       340       350
        400       410       420       430       440       450       460       470
ceDMWD FDPYLCTTEEDLENNGIAMTSTFDDVAKDFSIRSGPVPSTSADLNSGVMNATSTFSRCSLASFNTINGAPAGNSVRYRIG
       ::::   .::       : .:.  : . .   .. :   ::.:  .:.                 ..  : ..:. ::.:
mDMWD  FDPYTTRAEE------AASASADGDPSGE---EEEP-EVTSSD--TGA---------------PVSPLPKAGSITYRFG
       360             370       380           390                         400
        480       490       500       510       520       530       540       550
ceDMWD SVGHDTFLCLWDITEDMLNQGNIRRHRNSTIIAPMTTLEVQTNSLVGRLEDLQEVSPGGAGVNASSDSQSITNNHTTPRP
       :.:.:: .::::.:::.:.  .    :. :.  : :   .   :  .:  .   ..:  ... :..    ...  .  :
mDMWD  SAGQDTQFCLWDLTEDVLSP-HPSLARTRTL--PGTPGATPPASGSSRAGETG-AGPLPRSLSRSNSLPHPAGGGKAGGP
     410       420       430       440       450       460       470       480
        560       570           580       590       600       610       620       630
ceDMWD EKQKKKKFTKRLG-FSKFT---SGSSSATSNPVGGHKIGTLMNGASV-NSESSKKQNPGLISQISCCNETRMLGSKFCPG
       . . .  .: :. .:     . .: ..    :.:.:. .:.:  ::...: ..:.  :..   . ...:. .::
mDMWD  NASMEPGIPFSIGRFATLTLQERRDRGAEKEHKRYHSLGNISRGGSGGNSSNDKLSGPAPRSRL---DPAKVLGTALCPR
         490       500       510       520       530       540           550       560
             640       650       660       670       680       690       700       710
ceDMWD IRDVPMIEPLMCKKVSHDRLTVLEFREDCVVTACQEGYICTWGRPGRYQPKRDCINSPGTASPESGQKPSGSTSAMTSSY
       :.:::.:::.:::.....:::: : :::..:::::: ::::.:::.    ..   . : ::    ..:: :.
mDMWD  IHEVPLLEPLVCKKIAQERLTVLLFLEDCIITACQEGLICTWARPGKAFTDEETEAQAGQASWP--RSPSKSV-------
            570       580       590       600       610       620       630

            720       730       740       750
ceDMWD GYGSDALNGVPPSRSSSTYSNTASTSVYHRPTYAWQNAN
            ..:.    ::.  :. ..: :
mDMWD  ------VEGI----SSQPGSSPSGTVV------------
                  640       650
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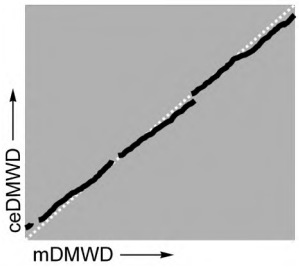


**B**

**Fig. 1.** Alignment of full-length ceDMWD and mDMWD proteins.
Panel A: Amino acid identity (:) and similarity (.) indicated on each line in between the ceDMWD and the mDMWD sequence. Numbers indicate amino acid residue positions in the respective proteins. The WD-repeat area of mDMWD is shown in bold. The peptide used to produce the antibody is underlined. Panel B: Matrix alignment of mDMWD (X-axis) with ceDMWD (Y-axis). A line straight from the left lower corner to the right upper corner (white dotted line) would represent a perfect match (100% identity). The black line is the alignment found for the full-length ceDMWD with the mDMWD protein.

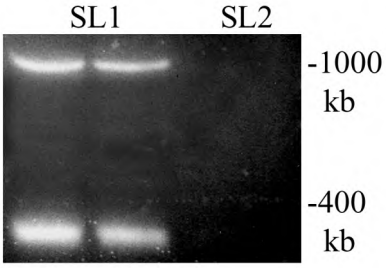


**Fig. 2.** RT-PCR DNA products of *trans*-spliced ceDMWD mRNAs carrying 5' SL1 or SL2 leader sequences. The SL1 primer gave two products of which only the 300bp was related to the ceDMWD sequence. RT-PCR with the SL2 primer did not produce any DNA products.

Since the transcriptional start site of the ceDMWD gene was positioned by computer prediction, it was not immediately clear if this gene was *trans*-spliced. To probe for presence of a leader sequence with SL1 or SL2 identity, we performed an RT-PCR followed by an PCR with either the SL1 or SL2 primer and a ceDMWD specific primer to amplify the pertinent ceDMWD 5'-region. In these experiments SL1 gave two DNA products of approximately 300 bp and approximately 1000 bp. SL2 did not give any products (Fig. 2). Both SL1 products were isolated and sequenced. The 1000 bp DNA band showed no relation to ceDMWD sequences. Sequence analysis of the 300 bp DNA band revealed use of the SL1 segment in the *trans*-splicing of ceDMWD mRNA, but in contrast to expectations, the SL1 sequence was not connected directely to the first predicted exon in ceDMWD (previously inferred from cDNA). Subsequently, alignment of the leader sequences with the genomic DNA sequence revealed presence of an additional exon and intron sequence in the ceDMWD genomic sequence (shown in a schematic drawing in Fig. 3).

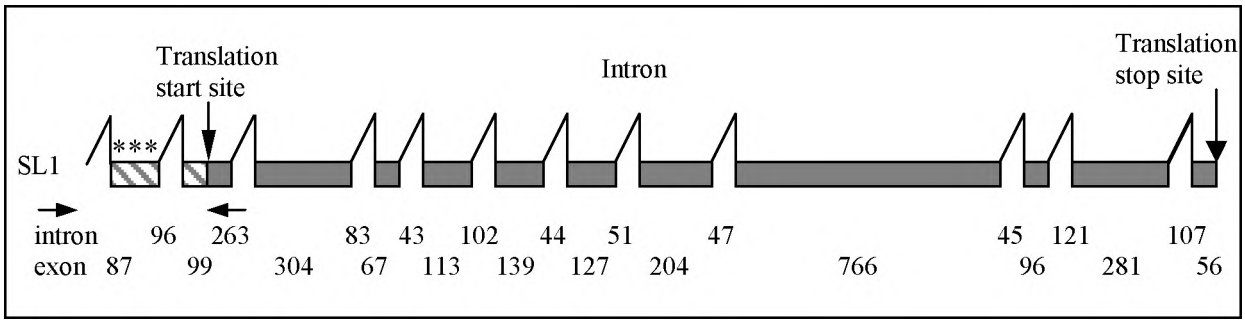


**Fig. 3.** Schematic overview of the entire transcription unit, SL1 *trans*-splice site and other intron/exon boundaries in the ceDMWD gene. Introns are depicted as tilted lines, exons as grey boxes and primers as horizontal arrows. The number of nucleotide residues in introns/exons is indicated. SL1 leaders are generally found immediately adjacent to the methionine start codon, however, SL1 is spliced further upstream (three possible translation start sites, indicated with asterisks) are located in between the SL1 and the proposed genuine translation start site (left vertical arrow).

In C. elegans, trans-splicing of SL leader segments tends to occur very close to the initiating methionine codon in mRNAs (often immediately adjacent), so the SL sequence may play a role in the translation initiation process [20]. We noticed, however, that the 5' ceDMWD mRNA sequence beyond the SL1 sequence contains three methionine codons. Presumably, these start codons should be considered non-functional because they are all followed by in frame stop codons within 10 to 50 bp. We must therefore assume that the fourth methionine codon is the actual functional translation-start site.

Yuji Koharas group also found, in a large cDNA screening of 65.000 clones, a cDNA fragment that included part of the first exon (clone yk408f5.5) of ceDMWD (http://nematode.lab.nig.ac.jp.). This confirmed that the mRNA sequence organization proposed here was indeed correct.

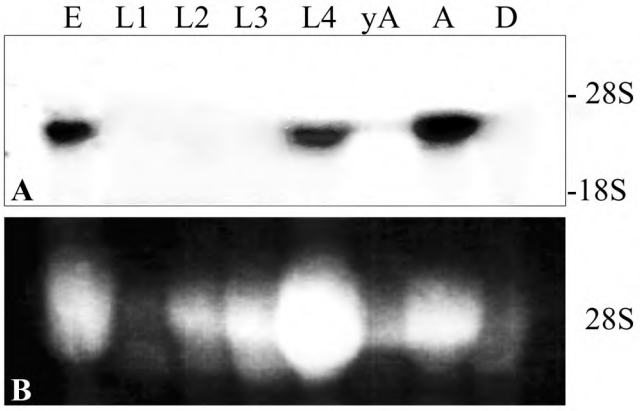


**Fig. 4.** RNA expression of ceDMWD in different developmental stages of *C. elegans*. Panel A: the ceDMWD signal: position of 28S (3.5 kb) and 18S (1.8 kb) ribosomal RNAs is indicated. Panel B: ethidium bromide stained 28S RNA, as control for loading.
E: embryos, L1: larval stage 1, L2: larval stage 2, L3: larval stage 3, L4: larval stage 4, yA: young adults with no embryos yet, A: adults with embryos, D: dauers.

*RNA expression*

To identify when during the life cycle of *C. elegans* ceDMWD RNA was expressed, we performed Northern blot analysis. For this purpose we isolated total RNA from embryos, L1, L2, L3, L4, young hermaphrodites (containing no embryos), gravid hermaphrodites (containing embryos) and dauers (diapause stage, formed when food supply is insufficient). Normalization to total RNA content was used to compare expression levels of the different developmental stages. Despite the uneven loading it was clear that the ceDMWD RNA (signal at approximately 3.2 kb, which was the predicted size) was expressed in embryos and gravid adults (Fig. 4). The L4 larval stage also showed RNA expression but when normalized to the total RNA, this expression level was quite low. None of the other developmental stages showed detectable ceDMWD RNA expression. We assume, as we found some expression in L4 larvea as well, that the expression seen in gravid adult *C. elegans* originated from both the embryos and the adults themselves.

To determine where in embryos and adults ceDMWD RNA is expressed, we performed RNA *in situ* hybridizations. These were done on isolated embryos and adult worms. The embryos were only ceDMWD positive in the early stages (one to six-ten cells) with the RNA randomly distributed over all cells (Fig. 5 panel A and C ). Older embryos (more than 10-15 cells) did not show any specific ceDMWD signal (Panel I). In adult worms the gonads were clearly ceDMWD positive, specifically in the mitotic area (distal part), the transition zone between mitotic and meiotic cells and the pachytene area (more proximal part) (Fig. 5E). The staining was found throughout the gonad with no specific preference for any of the named regions; each region showed the same level of expression.

*Protein expression*

To determine if the ceDMWD protein is also expressed in gonads and embryos and if so, where it is localized, we developed a polyclonal antibody against a ceDMWD peptide for use in indirect immunofluorescence (Fig. 6). Since tests on Western blots had shown that the antibody recognized the coupled peptide we tried to purify the polyclonal antibody using affinity purification using the peptide.

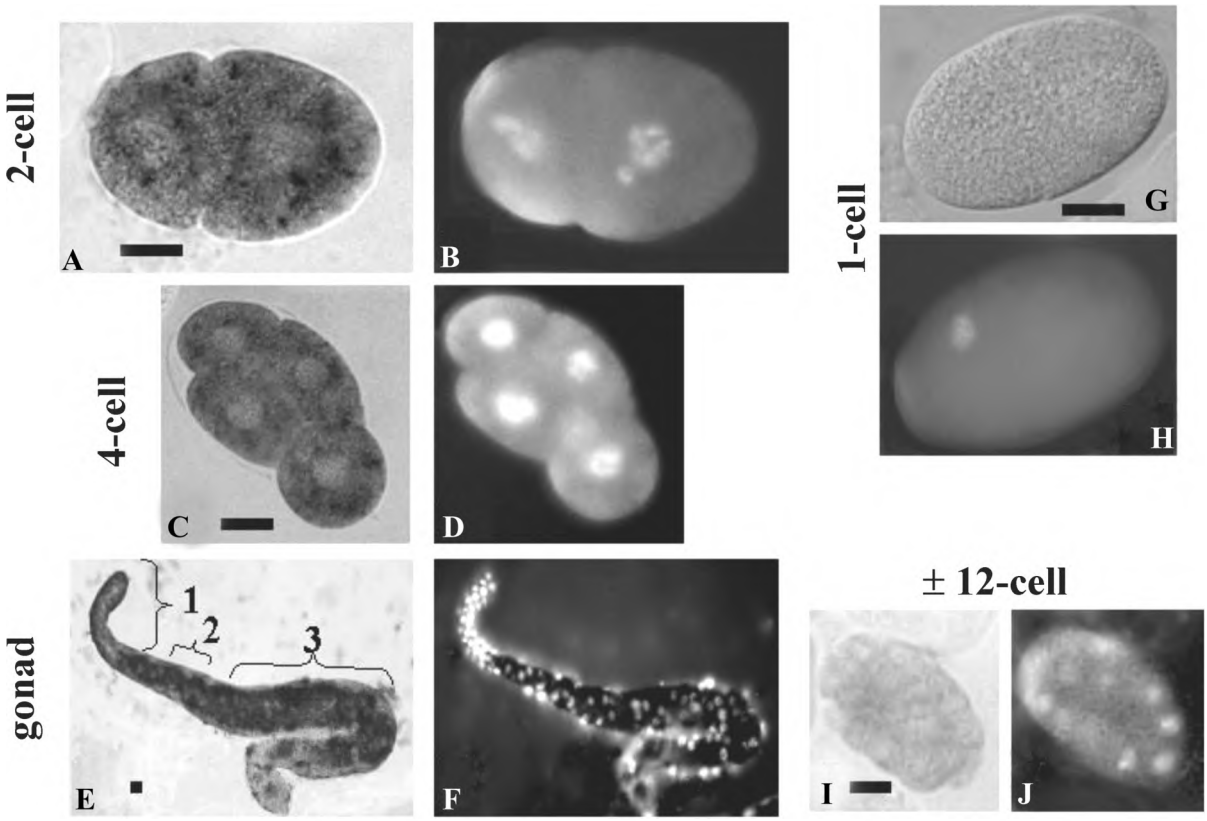


**Fig. 5.** RNA *in situ* hybridization with ceDMWD anti sense probe on embryos and gonads. Positive staining is shown in dark grey (throughout the embryos and gonad). Panel A: two-cell embryo stained with ceDMWD anti-sense probe. Panel B: DAPI staining of this two-cell embryo. Panel C: four-cell embryo stained with ceDMWD antisense probe. Panel D: DAPI staining of this four-cell embryo. Panel E: staining of wt gonad with ceDMWD anti-sense probe. Note that staining is throughout the gonad's distal mitotic region (no.1, upper left) the transition zone (no.2, middle) to the proximal pachytene region (no.3, lower right). Panel F: DAPI staining of this same gonad. As the ceDMWD staining is quite strong it blocks the DAPI staining. Panel G: One-cell embryo stained with sense ceDMWD. Panel H: DAPI staining of this same embryo. Gonad staining with sense did not show any signal (not shown, comparable to the panel G). Panel I: Approximately twelve-cell embryo incubated with the anti-sense probe showing no specific ceDMWD staining. Panel J: DAPI staining of this embryo. Bar represents approximately 10 μM.

However, no ceDMWD antibodies could be recovered after the purification procedure. Therefore we decided to use the non-purified serum, after clearance of antibodies against *E.coli* proteins for the immuno staining experiments.

As a control for each experiment we blocked the antibody with purified peptide to demonstrate its specificity (Fig. 6G). The immunostaining experiments revealed that ceDMWD protein is expressed in the gonads of adult worms and in early embryos (Fig. 6). The early embryos showed clear staining at all cell-cell membranes with sometimes clear dots of accumulated ceDMWD protein (Fig. 6A). We did not detect any difference in staining of the cell boundaries between cells of different lineages. The cortex of the embryos did stain for ceDMWD.

In gonads, ceDMWD expression showed a honeycomb pattern. Images at different focal planes indicated that the ceDMWD expression was either located at the outer membranes of the germ line cells or in the somatic sheath-cells that cover the gonads.

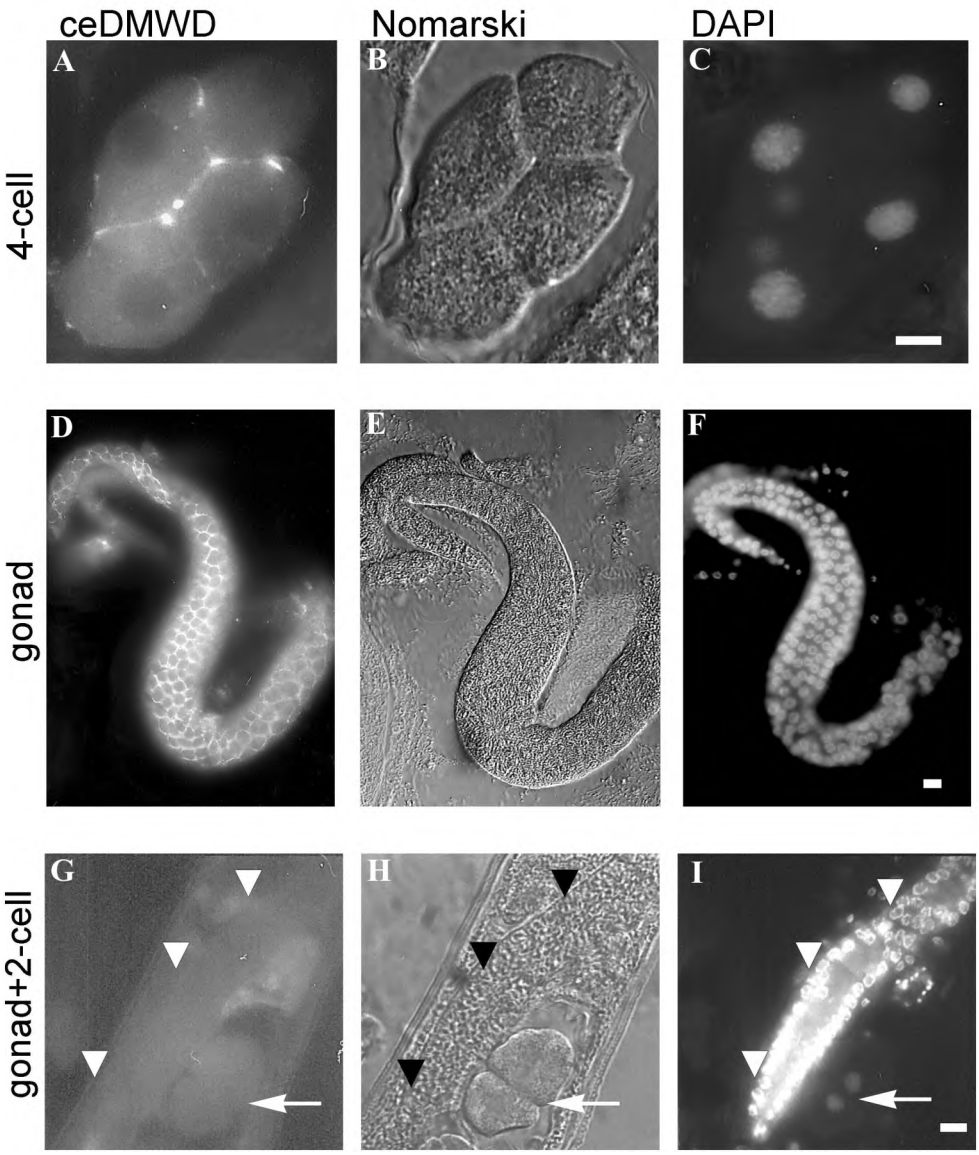


**Fig. 6.** Protein staining of embryos and gonad with ceDMWD antiserum.
Panel A: Staining of four-cell embryo with ceDMWD antibodies showing the cell-cell contacts to be stained. In the older embryos this staining was less pronounced. Panel B and C: Nomarski image and DAPI staining of this four-cell embryo respectively. Panel D: Antiserum staining of gonad revealing a honeycomb pattern. This is a superficial gonadal plane. In a central plane, staining is only seen at the periphery, suggesting that the ceDMWD is located on the outer surface or in structures in close vicinity of the gonad, but actually not within the gonads. Panel E: Nomarski view and panel F: DAPI staining of the gonad. Panel G: ceDMWD staining of gonad and 2-cell embryo incubated with purified peptide to block the ceDMWD signal. Note that ceDMWD signal is absent in the gonad as well as in the 2-cell embryo. Panel H and I: Nomarski view and DAPI staining of the same gonad and embryo. Arrow heads in G, H and I point at the gonad and arrow points at the cell membrane of the two-cell embryo. Bar represents approximately 10 μM.

To identify the exact cells that would express ceDMWD, we made transgenic *C. elegans* by coupling up to10 kb of the 5'-ceDMWD region to GFP and/or lacZ. However, none of these transgenic animals showed expression in either embryos or gonads.
It is known in C. elegans that transgenic DNA is silenced in the germ line and early embryos and that this silencing probably mechanistically relates to RNAi [210].

We therefore also produced ceDMWD transgenic worms in rde-2 deficient mutants, which are RNAi deficient and derepress germ line and early embryonic expression of transgenic arrays [210]. Unfortunately, again none of the transgenic lines were expressed in the germ line.

*RNA interference (RNAi)*

Presence of double-stranded RNA (dsRNA) induces sequence-specific loss of gene expression in *C. elegans* [51]. This phenomenon called RNA interference (RNAi) is likely a cellular defence mechanism that uses double-stranded RNA (dsRNA) as a sequence-specific trigger to guide the degradation of homologous single-stranded RNAs. It is a multistep process involving at least one type of RNA intermediate, a population of small 21-25 nt. RNAs (called siRNAs) that are initially derived from cleavage of the dsRNA trigger and several proteins like RDE-4 and RDE-1 [154,166].

To analyze the ceDMWD loss-of-function phenotype in *C. elegans* we used RNAi procedure on wild-type animals. Embryos of injected animals had no obvious defects. However, the F1 generation of the ceDMWD dsRNA injected mothers produced more dead embryos than the F1 generation of the TE injected controls (hermaphrodites were injected with TE and treated the same as the dsRNA injected animals). In these experiments we only counted the amount (number or percentage) of F1 mothers that gave rise to ≥ 10% dead embryos (Fig. 7A). At 20°C, 24% of the F1 mothers gave rise to ≥ 10% dead embryos (N=140) while in the control group this was 10% (N=100). At 25°C the effect was more pronounced; 39% of the F1 mothers gave rise to ≥ 10% dead embryos (N=348) while the percentage in the control group stayed the same (10%; N=62). When the distribution of the dead embryos was taken into account, the temperature sensitivity effect was more obvious (Fig. 7B). For instance the amount of dead animals between 41 and 50% at 25°C is 19 out of 348 (5.5%) compared to 1 out of 140 (0.7%) for the RNAi at 20°C. Note that in panel B only the F1 hermaphrodites that gave rise to > 10% dead embryos are shown.

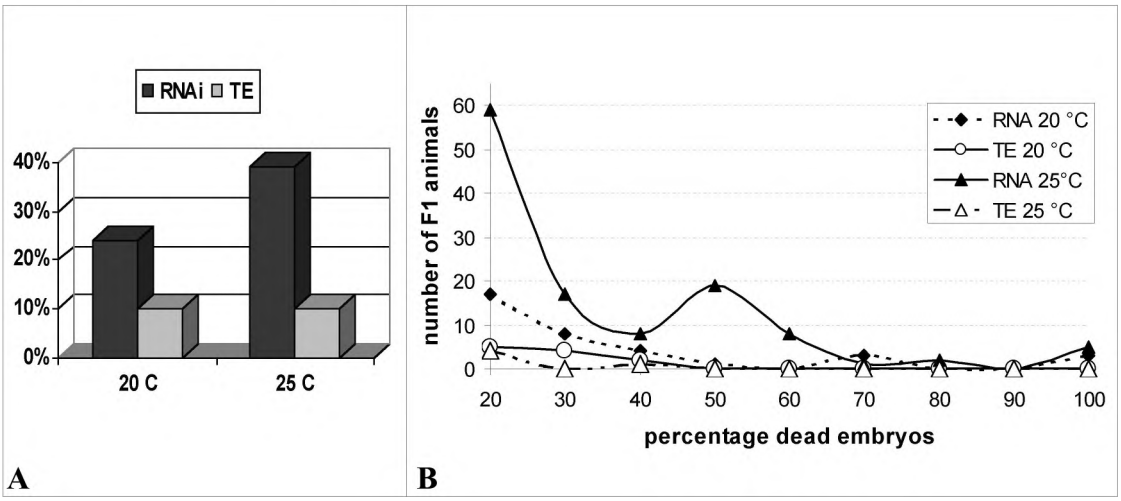


**Fig. 7.** Graph of the ceDMWD RNAi experiments.
Panel A: Graph of temperature sensitivity of ceDMWD treated hermaphrodites. Progeny of ceDMWD RNAi injected worms in black and progeny of TE injected worms in grey. The Y-axis shows the percentage of dead embryos. The X-axis shows the temperature at which the embryos were incubated. Panel B: Distribution of F1 hermaphrodites that gave rise to > 10% of dead embryos. F1 animals that gave ≤ 10% dead embryos are not shown, each point in the graph is the amount of F1 hermaphrodites in a group that gave rise to 11-20% (shown at 20 in the graph), 21-30%, 31-40% etc. dead embryos. Total numbers of hermaphrodites in each experiment RNAi 20°C: N=140, RNAi 25°C: N=348, TE 20°C: N=100, TE 25°C: N=64.

We noted that a percentage of the embryos did not hatch so we analyzed the lineages of early embryos (one to four cells) of worms that had been laying a high percentage of dead embryos by Nomarski microscopy. We did not detect any obvious defects in cell division patterns or cell membranes, which is the site of ceDMWD localization but about 50% of these embryos analyzed arrested after 2 to 5 hours (over 100 cell stage) post-fertilization, while the TE control embryos all hatched. Some of the arrested embryos developed until the comma stage (after approximately 6.5 hours of development, the stage in which the first invagination is visible and the embryo looks like a comma) but most arrested earlier. Unfortunately we could not infer a specific stage at which the embryos would arrest.

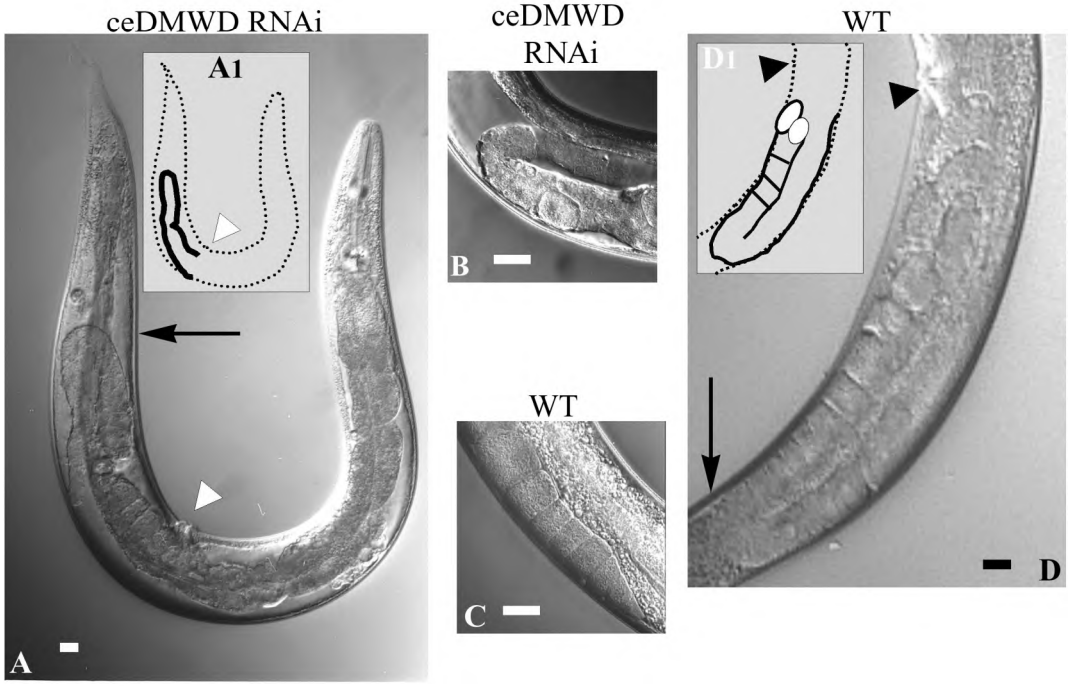


**Fig. 8.** CeDMWD RNA injected worms showing defects in the gonads.
Panel A: offspring of injected worm with gonadal defects. Panel $A_1$: artistic rendering of the defect shown in 8A, outlining the posterior gonad arm. Panel B: higher magnification of other offspring of ceDMWD RNA injected worm showing the gonadal arm detached from overlying tissue. Panel C: close up of gonad of wild type. Panel D: wild type with normal extended gonad in contact with the surrounding tissue. Panel $D_1$: artistic rendering of gonad in wild-type animals. TE injected animals produced normal looking (wild-type) offspring. Bar represents approximately 10 μM, arrowheads indicate the vulvas, arrow points indicate the distal ends of the gonads.

As a percentage of the F1 generation of injected worms would give rise to dead embryos, we also examined these worms microscopically. We found that in most cases (90%, N=40) defects in the gonads were apparent, compared to the low levels of defects seen in TE injected worms (5%, N=30). The defects worsened as the worms aged. In approximately 80% of analyzed worms the gonads were detached from the overlying tissue and the loop region was not situated as far anteriorly or posteriorly (for the anterior and posterior gonadal arms, respectively) as normal (Fig. 8). Oocytes were not found in a row but were randomly distributed through the gonad. These worms, however, produced some live offspring indicating that at least part of their gonads were still functional. In a few cases (10%) we also observed that the gonads appeared normal but the oocytes were smaller than normal (not shown). Taken combined, these observations indicate a gonadal defect and suggest a role for ceDMWD in the germ line development.

## Discussion

*Homology analysis*

In this chapter we analyzed the expression pattern and loss-of-function phenotype of DMWD in *C. elegans*, named ceDMWD. This gene is unique in the *C. elegans* genome and is the proposed orthologue of the mouse and human DMWD gene. In chapter 5 we identified orthologues of DMWD in the genomes of human, mouse, C. *elegans*, *A. nidulans*, *S. pombe*, *A. thaliana* and *D. melanogaster*. In Table 1 we reanalyzed the extend of DMWD homology between all possible combinations of species in this series, confirming the remarkable high evolutionary conservation of segments across the entire polypeptide between lower and higher eucaryotes. Overall alignments of ceDMWD with mDMWD revealed 33.1% amino acid identity and 58.8% similarity. The WD-repeat domains - that are thought to serve as a protein-protein interaction site - showed the highest similarity (68% similar) but there is also prominent homology across the C-terminal end of the protein. The high similarity between the full-length proteins suggests that they might have similar protein partners and work in an evolutionary (from plant to human) conserved pathway.

*RNA expression*

One feature that distinguishes DMWD mRNA in C. elegans from that in higher eucaryotes is the origin of its 5' leader sequence. Clear evidence was obtained (Fig. 2) that a SL1 leader segment is *trans*-spliced onto the body of the ceDMWD mRNA. *Trans*-splicing is quite common in *C. elegans* and occurs in about 70% of mRNAs expressed [19,20]. Our analysis revealed also that the 5' UTR of the ceDMWD mRNA is longer than predicted by computer alignment of known cDNAs and genomic sequences. This observation implies that ceDMWD forms an exception to the rule that splicing places the SL segment very close to the initiating methionine codon. From the 83 *trans*-spliced genes analyzed only 4 had their *trans*-splice site further away than 30 bp [20]. In ceDMWD we find 106 nucleotides between the end of the SL segment and the initiating start codon. As the exact function of the 5'-splice leaders in *C. elegans* mRNAs is not known, it is difficult to tell if this phenomenon confers any specific properties (e.g. regarding translatability or stability) to ceDMWD mRNA.

Northern blot and PCR analysis of mRNA expression during *C. elegans* development revealed that ceDMWD mRNA is expressed during both the embryonic and adult stage. No changes in mRNA structure and size were apparent, so we have no indication for alternative promoter use or splicing at different time points in development. *In situ* hybridization revealed that embryos expressed the ceDMWD RNA only when they were very early in development (1 to 6-10 cell stage). At this stage the RNA was expressed uniformly throughout the embryo (Fig. 5). We consider it therefore likely that the mRNA observed in the embryonic stage is derived from maternal expression degrading during embryonic development. In adult worms the ceDMWD RNA was uniformly expressed throughout the gonads rather than at the edges (Fig. 5), which suggests that the ceDMWD mRNA originates from the germ cells themselves rather than in the surrounding somatic sheath cells. When taken together our results suggest that ceDMWD mRNA is only functional in early embryos and just prior to or during germ cell formation.

*Protein expression.*

The ceDMWD protein, like the ceDMWD mRNA, was only seen in early embryos where it was mainly located at the cell-cell boundaries in 1 to 6-10 cell embryos. *C. elegans* embryos divide asymmetrically, starting with a polarized 1-cell embryo $P_0$, which divides into the larger anterior AB cell and into the smaller posterior $P_1$ cell.

Then, in turn both cells divide again with $P_1$ and its descendants dividing asymmetrically. It has been demonstrated that some proteins are localized in an asymmetric fashion in the early embryo. PIE-1 for example is located only in the P lineage [190] or GLP-1, which is expressed only in the AB daughters [112,185]. To check if ceDMWD was possibly involved in the polarization of the early embryo we analyzed 2 and 4 cell embryos (N=10) but there were no obvious differences between specific cell-cell boundaries, indicating an equal distribution of ceDMWD.
When we analyzed the specific localization of ceDMWD we also found dot-like structures at the cell-cell membranes (Fig. 6) but with microscopic analysis these structures could not be specifically assigned to descendants of A or P cells. In a few cases we even did see ceDMWD staining at the chromosomes in $P_1$ cells in prophase (not shown). Accumulation in dot-like structures (associated with spindle figures) and dynamic (re)distribution of protein in cytosolic and cortical patterns during early phases of embryogenesis has also been observed for other proteins, like actin or actin capping protein (CP), in *C. elegans* [223]. To know whether these similarities in protein location and assembly behaviour at cell-cell boundaries and prophase chromosomes indicate any functional relationship between ceDMWD, actin and CP more work has to be done.

In the hermaphrodite gonads ceDMWD staining was found in a honeycomb-like pattern, consistent with expression on the outside facing membrane of the germ cells. The staining was distributed like the ceDMWD RNA, with no preference for a certain cell-type along the gonadal proximal-distal axis. Moreover ceDMWD was equally expressed throughout the distal mitotic region, the transition zone between mitotic and meiotic cells and the meiotic pachytene region of the gonad (Fig. 6). The *C. elegans* gonads are composed of germ cells that are separated by their direct neighbours via incomplete membranes and connected by an open central syncytium core. This organization (membranes on the outside but not in the middle) will give a honeycomb-like staining pattern when the membranes are stained, suggesting that ceDMWD is located in or close to the germcells membranes. However, the surrounding somatic sheath cells that are closely apposed to the germ cells would give a similar pattern. Since the mRNA is present within the gonad, the protein we observed is also likely to be there. Our combined data suggest that ceDMWD is generated by the germ cells for deposition for development of the early embryo and zygotically in adults for the gonad/germ line development or proliferation.

*RNAi*
To determine the ceDMWD loss-of-function phenotype we performed RNAi on wild-type *C. elegans*. We tried different methods of delivering the dsRNA including soaking, feeding and injecting. In our hands the injection method gave the most consistent results for ceDMWD and therefore we used this method. We noted that the phenotype obtained by injection was partially temperature-dependent. At 25°C the number of dead embryos in F1 animals was almost twice that of that at 20°C (39 vs. 23%) but at both temperatures they were considerably higher than in TE injected hermaphrodites. Other groups have also noticed a temperature-sensitivity of RNA injected worms. For example, when embryos of *cdc-42* RNAi injected mothers were analyzed after culturing at 25°C, embryonic lethality and osmotic sensitivity was found in 100% of the embryos, at 15°C there was also 100% embryonic lethality but there was no osmotic sensitivity [68].

When we analyzed the embryos of worms that produced high embryonic lethality (>25%), we found that in most cases there was a correlation to morphological abnormality in the gonads (Fig. 7). The early embryos did not seem to have membrane defects, as might be expected on basis of the protein localization, but they did arrest after 2-5 hours. As the hermaphrodites with gonadal defects still produced some normal developing offspring, programming of germ line development and fertilization of the embryos was still partly functional. This could indicate that ceDMWD on its own is not absolutely essential for embryogenesis and germ line development but it is also possible that the penetrance of dsRNA inhibition was not complete and therefore some ceDMWD protein would still be produced. Two large-scale RNAi screens have shown that approximately 85% of *C. elegans* genes show no obvious phenotype but chances of observing a phenotype increase when the gene is evolutionary conserved [54,65].
Based on the findings described in this chapter we would like to propose that ceDMWD is the (functional) orthologue of mDMWD, with a possible role in pathways involved in embryogenesis and the germ line development of *C. elegans.* Recently, Lockington and co-workers have shown that *creB*, the homologue of mDMWD in *Aspergillus nidulans*, is involved in carbon catabolite repression, hence gene expression regulation [133,134]. If we combine these findings with our observations here on dynamic protein location, programming of germ line formation and early development in *C. elegans*, and then extrapolate this to the situation in higher eucaryotes, a putative role of DMWD in germ line development or early gene expression regulation in mouse and human should be studied more intensely.

# Chapter 7

## Summarizing Discussion

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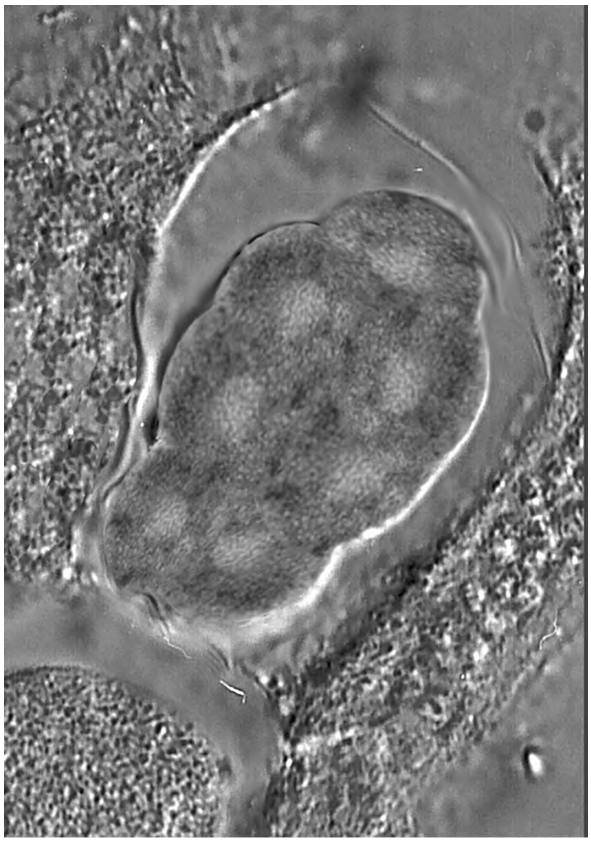

This Ph.D. study has provided us with improved insight into the role and fate of the DMWD protein in different tissues like brain (chapter 2) and testis (chapter 3) of mouse, as a model for the situation in humans. Moreover, by combining bio-informatics and wet-laboratory approaches knowledge was gathered on associating partners of DMWD (chapter 4) and the molecular structure (chapter 5). Finally, we studied the significance of an evolutionarily conserved DMWD-homologous product in an easily accessible model organism, *C. elegans* (chapter 6). All these investigations were performed to obtain clues about the biological function(s) of DMWD and its possible pathophysiological relevance in human DM patients.

In chapter 2 we focussed on DMWD expression in brain. We showed that DMWD RNA expression in vivo and in vitro was under differential developmental control and that expression of the DMWD protein (doublet at 70 kDa) was temporally regulated with a specific spatial distribution in neurons and in areas that contained many synaptic endpoints. The unique temporal regulation and spatial distribution of DMWD in the postnatal developing mouse brain, with the strong expression in brain areas with many synapses, suggested that DMWD has a very specialized function in neuronal development and specifically in the dendrites with their synapses. Brain related symptoms seen in DM1 patients are mental retardation, sleep disorders, behavioural changes and (progressive) alterations in brain structure and cognitive defects with serious consequences for social functioning [29,43,83]. Some indirect evidence has been provided suggesting that miss-splicing of tau gene products may be involved in these devastating aspects of the disease [191]. We conclude however from our work, based on the specific localization of the DMWD protein in the CNS, that DMWD could also be a good candidate for being involved in (some of) the neuropathological aspects of DM1. Based on theoretical considerations, one expects problems with DMWD expression to be most evident in cases with congenital DM1 and we would like to propose therefore that follow up studies should concentrate on the role of DMWD in this category of DM patients.
A putative partner protein that could be involved in the very specialized function of DMWD in brain was found in literature: the deubiquinating enzyme UBHI. UBHI is the human homologue of CreB, which is known to interact with CreC, the *A. nidulans* homologue of DMWD [134,217]. Although a role for UBHI in human brain function in DM patients is at this moment purely speculative it may be relevant to mention that a connection between ubiquitination-dependent mechanisms and synaptic growth was already described for *Drosophila,* indicating that synaptic development may be controlled by the balance between positive and negative regulators of ubiquitination [44]. Moreover, Wilson and co-workers suggested in their article about synaptic defects in ataxia mice that ubiquitin regulators (specifically ubiquitin proteases) are important in the regulation of synaptic activity in mammals [234]. Extrapolation of this information to the situation for DMWD in mouse/human may suggest that a role for DMWD (and UBHI as partner) must be sought in (transcriptional) regulation of the processes involved in growth and maturation of dendritic synapses. Reverse genetics studies, with gene knockout or siRNA mediated knockdown of DMWD expression in mouse models, will be necessary to validate this hypothesis.

In chapter 3 we demonstrate that DMWD protein in testis appeared as two distinct polypeptides, with molecular sizes of 78 and 37 kDa, while the DMWD mRNA appeared in one uniform size. When analyzed in more detail these proteins seemed to be expressed in different cells within the testis, the 37 kDa mainly in maturing Sertoli cells, where it might have a role in maturing Sertoli cells and the 78 kDa mainly in spermatocytes, including the sex vesicle. The sex vesicle is a very specialized structure in which the sex chromosomes are synapsed and it is only present during the pachytene stage [4,121,202].

The 78 kDa DMWD protein occurred also in elongating spermatids were it is possibly involved in meiosis and development of the spermatids. Together, these observations suggest a very specialized role for DMWD in meiosis, during the synapsed stage of the sex chromosomes.
As discussed above for brain, UBHI could be a partner protein for DMWD. For the specialized role in testis UBHI could be the interacting protein as well. Although an exact role for ubiquitinylation during meiosis is not clear, recently Baarends and co-workers showed that loss of a ubiquitin-conjugating enzyme (HRB6) leads to male infertility, caused by major changes in the synaptonemal complexes and increased crossing over frequencies [8].

However, in Chapter 4 we identify another possible interactor with a more clearly defined biological role in chromatin modification and compaction during gametogenesis. From a yeast two hybrid screening with DMWD as bait, the human heterochromatin protein 1 gamma ($HPI^{hs}\gamma$) was found as a possible binding partner for DMWD. $HPI^{hs}\gamma$ is part of a heterochromatin sub-family, which consists of three HPI proteins: $HPI^{hs}\alpha$, $HPI^{hs}\beta$ and $HPI^{hs}\gamma$ [152,173]. HPI proteins are chromatin-binding protein involved in position-effect variegation [188]. The three $HPI^{hs}$ variants are very similar in sequence and $HPI^{hs}\beta$ and $HPI^{hs}\gamma$ but not the $HPI^{hs}\alpha$ were shown to be concentrated in the sex vesicle during spermatogenesis [150], making HPI an excellent candidate for being a partner protein of DMWD in testis.

Although my findings in this Ph.D. study did not assign a direct function to DMWD, the fact that both putative DMWD interactors HPI and UBHI are involved in gene regulation strongly points to a possible role of DMWD in gene regulation. Whether such a role would be exerted indirectly via association in protein complexes with regulatory (ubiquinitating) activity (UBHI) or by direct interaction with DNA binding proteins (HPI) is still an open question.
Finally, it is of note that testis related DM1 symptoms involve testicular atrophy, which presents itself in small and soft testes with tubular degeneration and hypospermatogenesis. Based on our findings on DMWD in specialized compartments of testis cells, it is therefore tempting to speculate that DMWD is a good candidate gene to be involved in the testicular atrophy seen in DM patients with highly expanded $(CTG)_n$-repeats, most likely via its association to chromatin regulation in relation to germ-cell development.

Association of DMWD to HP1 may also have broader implications for the DM phenotype than revealed in Chapters 3 and 4. In a recent study HPI, the possible partner protein of DMWD, was namely also connected to muscle differentiation. In this work HPI(alpha) was shown to be associated with the MEF2-interacting transcriptional repressor (MITR), class II histone deacetylases (HDACs) and the histone methyltransferase (HMTase) SUV39H1 [240]. Class II HDACs are highly expressed in heart, brain, and skeletal muscle and are known to interact with myocyte enhancer factor 2 (MEF2), which regulates muscle differentiation (reviewed by [147]). SUV39H1 places a 'methyl marker' on histone H3, which is then recognized by HP1 [12]. The association of SUV39H1, class II HDAC and HPI provides an efficient mechanism for modifying nuclosomal histone tails and stabilizing the methylated state of histone H3 lysine 9 via the docking of HPI. The stability of these chromatin-remodelling complexes is subject to histone methylation in response to extracellular cues that alter intracellular calcium concentration and promote muscle differentiation [240].
It has been fairly well documented that Myotonic Dystrophy is not only characterized by muscle weakness and wasting, but also that delayed muscle maturation in infantile myotonic dystrophy is a distinct feature [58,94].

Typically, muscle fibres and/or cultured skeletal muscle cells of DM patients exhibit a decreased resting membrane potential and increased basal cytosolic $Na^+$ and $Ca^{2+}$ concentrations [15]. When DMWD is indeed associated with HPI and through this interaction is associated with SUV39H1 and class II HDAC, this would mean that DMWD could be a candidate for being involved in muscle development.

Findings presented in chapter 5 are particularly relevant for the supposed role of DMWD in transcriptional and differentiation-regulatory pathways. In this chapter, we focussed on the biochemical properties of the core domain of the DMWD protein, the WD-repeat subunits. We showed there that this domain is composed of four trypsin-resistant WD-repeats, with a linear sequence spacing, which is highly conserved during evolution. The four WD-repeats in DMWD are folded like a genuine β propeller structure, with high resistance against trypsin protease activity. The β propeller structure is very well designed for multiple reversible interactions, either sequential or with different partner proteins at the same time, including the top, the bottom, and even the outside of the propeller as possible binding sites [199]. For instance, the best-characterized WD-repeat proteins are the G β subunits of G-proteins. These G-proteins transduce signals across the plasma membrane by binding simultaneously to several different proteins or protein complexes. Therefore WD-repeats, including DMWD, are very well suited for coordinating sequential and/or simultaneous dynamic interactions involving several (sets of) proteins, which makes WD-repeat proteins excellent regulatory proteins that can be active in diverse cell compartments.

Another further clue for the biological function of DMWD came from the *C. elegans* homologue (ceDMWD) examined in chapter 7. The expression distribution of ceDMWD gene products, ceDMWD protein and mRNA occur in early embryos and adult gonads, was somewhat reminiscent to that in mice where DMWD is also prominently expressed early in development and in (adult) testis. Our experiments analysing the effects of ceDMWD loss-of-function showed that progeny of injected worms had gonadal defects and part of the offspring of the F1 generation arrested, mostly before mid-embryogenesis. These findings led us to propose that ceDMWD is involved in early embryogenesis and germ line development. Also, DMWD's role in early development could be either through a gene regulation pathway by direct interaction with the DNA binding protein HPI or in a ubiquinitating pathway (UBHI). Both proteins do have a putative homologue in *C. elegans* [34,80] and recently Couteau and co-workers showed that a *C. elegans* HPI homologue is involved in germline development [34], however further research is required to investigate the pathway in which ceDMWD is involved.

The widespread occurrence of DMWD throughout the eucaryotic kingdom, its broad expression range, the different molecular weights (hence molecular surroundings) in brain and testis, and its various subcellular locations suggest that DMWD might have more than one biological function or has a very dynamic regulatory role.

During the process of this thesis many clues for the biological function(s) of DMWD were obtained, though nothing irrefutable. Therefore, future research with knockout or knockdown of function in cell and animal model systems is necessary before we can draw any definitive conclusions regarding DMWD's intriguing role in CNS, testis and various other tissues in health and disease.

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# Dutch summary
# Nederlandse samenvatting

# Dutch summary for laypersons
# Nederlandse samenvatting voor leken

# Nederlandse samenvatting

Het promotie onderzoek beschreven in dit proefschrift heeft ons een beter inzicht gegeven in de lokalisatie en de rol van het DMWD eiwit in de verschillende weefsels zoals brein/hersenen (hoofdstuk 2) en testis (hoofdstuk 3). Daarbij hebben we door het combineren van bio-informatica en "nat-laboratorium" onderzoek, informatie gekregen over partner eiwitten van DMWD (hoofdstuk 4) en de moleculaire structuur van het eiwit (hoofdstuk 5). Als laatste hebben we gekeken naar een evolutionair geconserveerde DMWD homoloog in *C.elegans*. Al deze onderzoeken werden gedaan om aanwijzingen te verkrijgen over de biologische functie(s) van DMWD en de mogelijke pathofysiologische relevantie van dit eiwit in Myotone Dystrofie (DM1) patiënten.

In hoofdstuk 2 hebben we ons gericht op DMWD expressie in brein. We hebben aangetoond dat DMWD RNA vanaf het begin van de ontwikkeling van hersenen/neuronen aanwezig is, terwijl de expressie van het DMWD eiwit hoger werd naarmate de ontwikkeling vorderde. Het eiwit werd gelokaliseerd op zeer specifieke plekken, plekken waar veel synapsen aanwezig zijn, wat kan betekenen dat DMWD in de ontwikkeling en de uiteindelijke werking van neuronen een hele specialistische functie heeft. Hersen-gerelateerde symptomen zoals worden waargenomen bij DM-patienten betreffen mentale retardatie, slaap stoornissen, gedrags veranderingen, veranderingen in de structuur van de hersenen en cognitieve defecten met serieuze consequenties voor het sociale functioneren van de patient. Op basis van het onderzoek beschreven in dit hoofdstuk concluderen we, mede gezien de specifieke localisatie van het eiwit in hersenen, dat DMWD een goede kandidaat is voor betrokkenheid bij (sommige) neuropathologische aspecten van DM1.
Een mogelijke partner voor DMWD in hersenen werd in de literatuur gevonden: het de-ubiquinerings enzym UBHI. Dit enzym is betrokken bij de regulering van genen door middel van ubiquitine. Door koppeling van de gegevens van deze twee eiwitten (DMWD en UBHI) kwam er een interessante mogelijke functie voor DMWD naar voren: het reguleren van processen die te maken hebben met de groei en ontwikkeling van neuronen en met name de dendritische synapsen.

In hoofdstuk 3 hebben we ons gericht op DMWD in testis. Het DMWD eiwit in testis komt tot expressie in twee vormen, een 37 kDa en een 78 kDa vorm. Het 37 kDa eiwit komt waarschijnlijk meer voor in Sertoli cellen, waar het mogelijk een functie heeft in de rijping van de Sertoli cellen. Het 78 kDa eiwit komt waarschijnlijk meer voor in spermatocyten inclusief de sex-vesicles. Het sex-vesicle is een zeer gespecialiseerde structuur, waarin de sex-chromosomen met elkaar paren. Daarnaast kwam het 78 kDa ook tot expressie in geëlongeerde spermatiden waar het mogelijk een zeer specialistische rol speelt in de meiose en de ontwikkeling van spermatiden. Mannelijke DM1 patienten hebben vaak kleine en zachte testikels met tubulaire degeneratie en hypospermatogenese. Gezien de zeer specifieke localisatie van de DMWD eiwitvormen is DMWD ook een goede kandidaat voor betrokkenheid bij (sommige) testis gerelateerde aspecten van DM1.

In hoofdstuk 4 hebben we ons gericht op eiwitten die mogelijk binden aan en samenwerken met het DMWD eiwit. Uit deze studie kwam één eiwit naar voren als mogelijke partner: heterochromatine eiwit I (HPI). Het is bekend dat HPI, net als UBHI, betrokken is bij het proces van genregulatie, wat een tweede (indirekte) aanwijzing vormt voor een rol van DMWD bij dit proces.

Een van de meest zichtbare symptomen van DM1 patienten is spierzwakte (vnl. in gelaatspieren en distale spiergroepen), spierverval, maar ook uitgestelde spierontwikkeling bij zwaar aangedane (meestal congenitale) patienten. In de literatuur word HPI ook gekoppeld aan spierontwikkeling. Als DMWD inderdaad interacteert met HPI, zou het ook een functie kunnen hebben bij spierontwikkeling en daarmee ook een rol bij de spier gerelateerde symptomen die- in met name congenitale - DM1 patienten voorkomen.

In hoofdstuk 5 hebben we ons gericht op de biochemische eigenschappen van het DMWD eiwit. De kern van het DMWD eiwit wordt gevormd door 4 WD-repeats, welke zeer geconserveerd zijn gebleven gedurende de evolutie. De WD-repeats zijn gevouwen in een β-propeller structuur, met een hoge resistentie tegen trypsine. Door de structuur van de WD-repeat eiwitten zijn ze goed uitgerust voor (simultane) interacties met een of meer eiwitten.

In hoofdstuk 6 hebben we ons gericht op de functie van DMWD in *C.elegans* (ceDMWD). *C.elegans* was gekozen als model-organisme omdat het zeer toegankelijk is. Het ceDMWD RNA komt tot expressie in vroege embryos en volwassen wormen. Bij het (gedeeltelijk) uitschakelen van het gen/eiwit zagen we dat de nakomelingen defecten hadden aan de geslachtsorganen (gonads) en dat de ontwikkeling van sommige embryos al in een vroeg stadium stopte. Ook in dit modelsysteem, net als in de muis, werden aanwijzingen verkregen die duiden op een rol voor DMWD in de ontwikkeling van de geslachtsorganen en in vroege embryogenese.

DMWD komt voor in veel eukaryote organismen (van schimmel tot mens) en heeft een breed scala aan weefsels waar het tot expressie komt. Dit kan betekenen dat DMWD meer dan één biologische functie heeft in het lichaam of één zeer dynamische regulerende functie heeft.
Gedurende het onderzoek beschreven in dit proefschrift, werden er verschillende aanwijzingen gevonden met betrekking tot de mogelijke rol van DMWD, maar niets is onomstotelijk vastgesteld. Daarom is het nodig om vervolgonderzoek te doen, mogelijk met knockout of knockdown muizenmodellen, zodat er meer bekend wordt over de functie van DMWD in hersenen, testis en verschillende andere weefsels, in gezonde mensen en in DM-patienten.

# Nederlandse samenvatting voor leken

Op verzoek van een aantal mensen, die eigenlijk toch wel eens wilden weten wat ik nu al die tijd van mijn promotie gedaan heb, zal ik proberen samen te vatten wat er in dit proefschrift staat. Hier wordt het onderzoek beschreven naar het Myotone Dystrofie WD-repeat (kortweg DMWD) eiwit en gen. Eiwitten zijn de werkmoleculen en bouwstenen van de cel, met een eigen plaats en functie. Een cel is de kleinste "levende" eenheid van de organen en weefsels in ons lichaam. Eiwitten zijn opgebouwd als lange ketens van bouwstenen, aminozuren genoemd, waarvan er twintig soorten in de natuur voorkomen. Door de schier eindeloze combinatiemogelijkheid van volgordes van aminozuren kunnen binnen cellen miljoenen verschillende eiwitten worden gevormd. De volgorde van aminozuren voor ieder type eiwit ligt echter wel vast en bepaalt hoe het eiwit eruit komt te zien en wat het doet. Feitelijk is de volgorde van inbouw van aminozuren in de ketens van eiwitten al vastgelegd in een soort code in ons erfmateriaal, het DNA (de afkorting voor deoxyribonucleïnezuur). Deze code voor eiwitvorming is eigenlijk heel simpel bepaald door de volgorde van de bouwstenen in dit DNA, waarvan er slechts vier verschillende zijn, met A, G, C of T aangeduid. Een volgorde van DNA bouwstenen (A's, T's, G's of C's) op het DNA met een functionele betekenis, noemen we een "gen". Ieder gen is dus de blauwdruk voor één of meer eiwitten. Genen liggen aaneengeregen verspreid over de DNA. De DNA-volgorde van een gen wordt als het ware afgelezen en vertaald (via een tussenstap) in een eiwitvolgorde. Is de keten van aminozuren – de eiwitketen - eenmaal gevormd, dan wordt het op een bepaalde manier ook nog eens ruimtelijk gevouwen, waarna het eiwit zijn uiteindelijke functie kan gaan vervullen (zie Fig. 1).

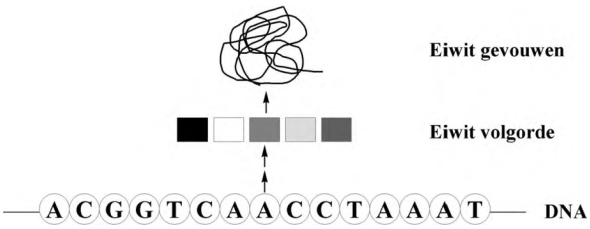


**Fig. 1.** Schematische weergave hoe eiwitten worden gemaakt.
DNA-volgorde wordt afgelezen en uiteindelijk vertaald in een aminozuur volgorde (een eiwitketen). Deze eiwitketen wordt op een bepaalde manier gevouwen, waarna het eiwit zijn functie kan gaan vervullen

Als er in het DNA en daardoor in het eiwit fouten zitten, kan het eiwit zijn werk niet meer goed doen. Ook kan er te veel of te weinig werkend eiwit worden gemaakt, waardoor er bepaalde processen in een cel verstoord kunnen raken en iemand ziek wordt.

Zoals eerder gezegd, wordt in dit proefschrift het onderzoek beschreven aan het Myotone Dystrofie WD-repeat (kortweg DMWD) eiwit en gen. Dit DMWD gen is interessant vanwege zijn plek op de DNA. Het DMWD gen ligt in een gebied waar veel genen dicht bij elkaar liggen en één van die genen is het Myotone Dystrofie Proteine Kinase (DMPK) gen. Dit laatste gen is betrokken bij de ziekte Myotone Dystrofie, doordat er een extra stuk DNA, een herhaalde volgorde met alleen C, T en G, in de DNA volgorde van dit gen zit. Dit zorgt voor veel problemen in het lichaam van Myotone Dystrofie patienten (DM1-patienten), waaronder spierzwakte (vnl. in gelaatspieren), spierverval, vertraagde ontspanning van de spieren, vertraagde ontwikkeling, mentale achterstand, leerproblemen, slaapstoornissen, kleine en zachte testikels, voortplantingsproblemen en staar.

Myotone Dystrofie patiënten kunnen een of meer van deze symptomen hebben en ook de ernst van de symptomen kan nog sterk verschillen. De ziekte wordt overgedragen van generatie op generatie en de symptomen worden vaak versterkt en treden eerder op naarmate het aantal generaties toeneemt. Bijvoorbeeld, oma is 70 en heeft alleen last van staar (last van haar ogen gekregen toen ze 55 was), terwijl haar dochter last heeft van vertraagde onspanning van de spieren (zo rond haar puberteit begon ze hier last van te krijgen) en haar kleindochter (drie jaar) al last heeft van spierverval, mentale achterstand, leerproblemen, slaapproblemen etc.

Een van de theoriën met betrekking tot dit zeer gecompliceerde ziektebeeld is dat – door het extra stuk DNA met alleen C's, T's en G's - niet alleen het DMPK gen zelf betrokken is bij deze ziekte, maar eventueel ook de genen die bij dit gen in de buurt liggen, bijvoorbeeld het DMWD gen.

In het onderzoek aan het DMWD gen hebben we (er zijn meer mensen betrokken bij zo´n onderzoek, vandaar "we") voornamelijk gekeken naar weefsels waarin DMWD het meeste voorkomt: in hersenen (hoofdstuk 2) en testikels (hoofdstuk 3). Ook hebben we gekeken of we eiwitten die binden aan of samenwerken met DMWD konden achterhalen (hoofdstuk 4), met het motto "aan zijn vrienden herkent men de man". Daarnaast hebben we ook gekeken hoe het eiwit er ruimtelijk uitziet (hoofdstuk 5). Als laatste hebben we het DMWD gen/eiwit in de worm *C. elegans* onderzocht (hoofdstuk 6). Dit omdat *C. elegans* als modelorganisme zeer toegankelijk is; het heeft een snelle voortplanting (binnen drie dagen heb je al zo´n 100 tot 200 nakomelingen) en er zijn veel manieren bekend om onderzoek te doen aan deze worm.

In het onderzoek aan hersenen zagen we dat het eiwit op een zeer specifieke plek in de hersenen aanwezig is, namelijk in de neuronen (cellen die zorgen voor de signaaloverdracht in de hersenen) en met name in de uiteinden van de neuronen (synapsen genaamd). Neuronen staan met elkaar in verbinding door middel van deze synapsen, de synaps van de ene neuroon geeft het signaal door aan andere neuronen en stuurt zo processen in het brein. Dit geeft aan dat DMWD dus een specialistische functie zou kunnen hebben in de hersenen. Myotone Dystrofie patienten kunnen een aantal problemen (symptomen) hebben die met de hersenen te maken hebben, zoals mentale achterstand, leerproblemen en slaapstoornissen. Deze problemen zouden verklaard kunnen worden (doordat DMWD op zo´n specifieke plek was gevonden) door een niet of verkeerd werkend werkend DMWD eiwit. Daarom concluderen we dat DMWD verantwoordelijk zou kunnen zijn voor een deel van de Myotone Dystrofie symptomen en dus betrokken zou kunnen zijn bij de ziekte Myotone Dystrofie.

In het onderzoek in testikels zagen we dat het DMWD eiwit op een zeer specifieke plek in de testikels aanwezig is. Dit betekent dat DMWD ook in testikels een heel specialistische functie heeft. Symptomen die we bij mannelijke Myotone Dystrofie patienten zien, zijn kleine en zachte testikels en problemen bij de voortplanting, door verminderde aanmaak van spermatozooen. Deze symptomen zouden verklaard kunnen worden door een veranderde werking van het DMWD eiwit en daarom concluderen we dat DMWD mogelijk ook betrokken is bij de testis-gerelateerde symptomen in mannelijke Myotone Dystrofie patienten.

In het vierde hoofdstuk hebben we gekeken naar eiwitten die samenwerken met het DMWD eiwit. Als we een partnereiwit zouden vinden waarvan bekend is wat het doet, zouden we zo een aanwijzing kunnen krijgen voor wat het DMWD eiwit in de cel doet. In dit hoofdstuk beschrijven we de vondst van één eiwit (heterochromatine eiwit of kortweg HPI) dat mogelijk samenwerkt met DMWD. Dit HPI eiwit is door een andere onderzoeksgroep op precies

dezelfde (zeer specifieke) plek gevonden in testikels als het DMWD eiwit. Dit maakt het dus zeer aannemelijk dat DMWD en dit HPI inderdaad samenwerken. Over HPI is al vrij veel bekend en men weet dat het eiwit bepaalde genen aan- en/of uitzet.
Een andere onderzoeksgroep in Australie heeft gekeken naar hetzelfde DMWD gen in een bepaalde zwam. Zij hebben laten zien dat het DMWD eiwit in die zwam samenwerkt met een eiwit UBHI genaamd. Ook UBHI heeft te maken met het aan- en/of uitzetten van bepaalde genen. Dus beide eiwitten (HPI en UBHI) die mogelijk samenwerken met DMWD, hebben te maken met het aan en uitschakelen van genen (het reguleren van genen). Dit is een zeer sterke aanwijzing dat ook DMWD te maken heeft met de regulatie (aan/uit-zetten) van bepaalde genen en zegt dus iets over de functie van DMWD in gezonde personen.

Verder hebben we gekeken naar hoe de ruimtelijke structuur van het eiwit er uit zou kunnen zien. Dit kun je doen door te kijken naar het DMWD eiwit en dit te vergelijken met de eiwitten die (voor bepaalde gebieden) zeer sterk op dat eiwit lijken. Soms kun je aan de hand van een dergelijke analyze ook iets zeggen over de functie (wat doet het eiwit) van jouw eiwit. In hoofdstuk 5 hebben we wel kunnen achterhalen hoe het eiwit er waarschijnlijk ruimtelijk uit ziet, maar we hebben hier geen algemeen geldende functie aan kunnen hangen.

Als laatste hebben we onderzoek gedaan aan het DMWD eiwit in de worm *C. elegans* (dit onderzoek is uitgevoerd in Calgary, Canada). We hebben gekeken waar het eiwit in de worm voorkomt; is het vergelijkbaar met waar het in de mens wordt gevonden ? We laten in hoofdstuk 6 zien dat het DMWD eiwit aanwezig is in de voortplantingsorganen van de worm, vergelijkbaar met de testikels in de mens. Als we het eiwit (gedeeltelijk) uitschakelen zien we dat de worm problemen krijgt met de voortplanting en dat de voortplantingsorganen bepaalde afwijkingen vertonen (vergelijkbaar met de voortplantingsproblemen die mannelijke Myotone Dystrofie patiënten ondervinden). Alweer vormt dit een aanwijzing dat DMWD mogelijk met de ziekte Myotone Dystrofie te maken heeft.

Misschien is het al opgevallen dat er heel vaak “mogelijk” en “zou kunnen” in deze samenvatting staat. Dit heeft te maken met het feit dat er wel aanwijzigingen zijn gevonden in dit onderzoek naar DMWD, maar geen direct bewijs is verkregen. We kunnen **aannemelijk** maken dat DMWD in ieder geval gedeeltelijk medeverantwoordelijk is voor het ziektebeeld van Myotone Dystrofie en dat het mogelijk te maken heeft met het aan en/of uitzetten van genen, maar echt hard bewijs hebben we niet kunnen leveren. Daarom is het nodig dat er vervolgonderzoek gedaan wordt, zodat er meer bekend wordt over de functie van DMWD (wat doet het precies), in gezonde mensen en in Myotone Dystrofie patiënten.

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## Dankbetuiging

## Curriculum vitae in English
## Curriculum vitae in het Nederlands

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## Curriculum vitae in English

The writer of this thesis was born on April 29, 1972 in Vinkel (part of the government of Geffen). In 1990 she received her high school diploma at Rodenborch college in Rosmalen (the Netherlands). After this she started her academic study in the Bioprocestechnology program, at the University for Agriculture in Wageningen (LUW). During this study she completed four practical periods (of six months each). Two within the Netherlands one at the department of Virology of the LUW (Prof. J. Vlak) the other at the department of Genetics (Dr. P. de Boer) and two abroad, one in China at the department of Virology of the Chinese Academy of Sciences in Wuhan (with Dr. Z. Hu as supervisor) the other in the United Kingdom at the department of Development Genetics of the National Institute for Medical Research, (N.I.M.R.) in London (with Dr. P. Burgoyne as supervisor). In January 1996 she graduated from the University and went to work for two months at the department of Development Genetics (with Dr. P. Burgoyne as supervisor) in London. In April 1996 she became a PhD student at the department of Cell biology at the University of Nijmegen (with Prof. B. Wieringa as supervisor). In 2000 she performed research on the *C. elegans* organism at the department of Biochemistry and Molecular Biology in Calgary, Canada (under supervision of Dr. P. Mains), for 8 months. As a PhD student she was active in several committees like the Medical PhD students Association and the Educational Committee of the Institute of Cellular Signalling (ICS). From September 2001 till March 2002 she worked at the labelling department of Organon Teknika. Since April 2002 she has been working at Diosynth at the Quality and Regulatory Unit in the Regulatory Affairs department, as scientific project leader for the Biochemical/Biotechnological products.

## Curriculum vitae in het nederlands

De schrijfster van dit proefschrift werd geboren op 29 april 1972 te Vinkel (gemeente Geffen). In 1990 behaalde zij het VWO diploma aan het Rodenborch college te Rosmalen, waarna zij aan de studie Bioprocestechnologie aan de toenmalige Landbouw Universiteit Wageningen (LUW) begon. Tijdens deze studie, voltooide zij vier afstudeervakken (van zes maanden), waarvan twee binnen Nederland, één bij de vakgroep Virologie van de LUW (Prof. J. Vlak), één bij de vakgroep Genetica (Dr. P. de Boer) en twee in het buitenland; één in China bij de afdeling Virologie van de Chinese academie van de wetenschap in Wuhan (onder begeleiding van Dr. Z. Hu) en één in Engeland bij de afdeling ontwikkeling Genetica van het nationaal instituut voor medisch onderzoek (N.I.M.R.) in Londen (onder begeleiding van Dr. P. Burgoyne). In Januari 1996 behaalde zij haar doctoraalexamen, waarna zij nog een korte tijd onderzoek verichtte aan de afdeling Onwikkelingsgenetica te Londen (onder begeleiding van Dr. P. Burgoyne). In april 1996 trad zij in dienst bij de afdeling Celbiologie, Faculteit der Medische Wetenschappen van de Katholieke Universiteit Nijmegen (onder leiding van Prof. Dr. B. Wieringa) als “assistent in opleiding”. In 2000 verbleef zij ongeveer 8 maanden in Calgary, Canada op de afdeling Biochemistry and Molecular Biology onder leiding van Dr. P. Mains, om te werken met het *C. elegans* organisme t.b.v. het proefschrift. Tijdens haar promotie was ze actief in het Medisch AIO Overleg en was ze lid van de AIO commissie van de onderzoeksschool “Insititute of Cellular Signaling”. Vanaf september 2001 werkte zij bij Organon Teknika op de afdeling labeling. Sinds april 2002 werkt zij bij Diosynth op de Quality and Regulatory Unit afdeling Regulatory Affairs als wetenschappelijk projectleider Biochemische-Biotechnologische producten.