Expression and function of the frontotemporal dementia gene *C9ORF72* in neuronal cells and human brain tissue

Stina Leskelä

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Faculty of Health Sciences

School of Medicine

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University of Eastern Finland, Faculty of Health Sciences

Program of Health Biosciences

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ABSTRACT

Frontotemporal dementia (FTD), which causes atrophy in the frontal and temporal lobes of the brain, is the second most common type of dementia within the working-age population. Recently, the major genetic cause behind this disease entity was found to be a mutation in the gene *C9ORF72*, which leads to a hexanucleotide repeat expansion in the noncoding region of the gene. The main goal in this Master's thesis study was to elucidate the expression and function of *C9ORF72* gene in normal conditions and proteasomal stress in the SH-SY5Y human neuroblastoma cell line and human brain samples. Overexpression of both protein isoforms A and B, and proteasome inhibition were utilized to elucidate the expression and function of the C9orf72 and other FTD-associated proteins. Levels of these proteins were also determined in the brain tissue samples of patients with or without the *C9ORF72* hexanucleotide repeat expansion. Here, the aim was to determine whether possible alterations in the levels of any of these proteins correlate with the presence of the pathological repeat expansion. Our results show significantly increased levels of C9orf72 isoforms after proteasome inhibition, indicating that C9orf72 protein levels are regulated by proteasomal degradation. Overexpression of isoform A, but not isoform B, specifically decreased the levels of endogenous ubiquilin-1 protein in SH-SH5Y neuroblastoma cell line implying that the two isoforms may have a different interactome. We did not find significant correlation in the levels of the investigated proteins and presence of the pathological repeat expansion in brain samples. However using a commercially available antibody, we detected slightly decreased levels of isoform B, but these results need yet to be confirmed wih another antibody. This Master's thesis provides novel data on the regulation and interaction of the C9orf72 proteins and effects of the pathological hexanucleotide repeat expansion in *C9ORF72*, even though further studies are required to elucidate physiological and pathophysiological effects of *C9ORF72*.

TIIVISTELMÄ

Frontotemporaalidementia (FTD) aiheuttaa aivokudoksen surkastumista otsa- ja ohimolohkoissa ja sen on todettu olevan toiseksi yleisin dementian aiheuttaja työikäisillä. Hiljattain *C9ORF72*–geenistä löydetyn heksanukleotiditoistojakson monistuman on todettu olevan yleisin perinnöllisen FTD:n aiheuttaja. Tämän pro gradu -työn tavoitteena oli selvittää *C9ORF72*-geenin ilmentymistä ja toimintaa SH-SY5Y neuroblastoma solulinjassa sekä ihmisen aivokudosnäytteissä. Soluja kasvatettiin joko normaaleissa kasvuolosuhteissa tai niitä altistettiin proteasomaaliselle stressille. *C9ORF72*:n proteiini-isoformi A:n ja B:n yli-ilmentymistä sekä proteasomaalista inhibitiota käytettiin tutkimaan geenin ilmentymistä sekä sen tuottamien proteiinien ja muiden FTD:aan liittyvien proteiinien tasoja. Proteiinitasoja mitattiin myös *C9ORF72*-toistojaksomonistuman kantajilta ja henkilöiltä, jotka eivät kantaneet toistojaksomonistumaa perimässään. Tässä tavoitteena oli tutkia vaikuttaako patologinen toistojaksomonistuma tutkittujen proteiinien tasoihin aivonäytteissä. Tulokset osoittivat, että sekä isoformi A:n, että isoformi B:n proteiinitasot olivat tilastollisesti merkitsevästi korkeammat soluissa proteasomi-inhibiittori käsittelyn jälkeen. Tämä tulos viittaa siihen, että C9orf72 proteiini-isoformien tasoja säädellään ainakin osittain proteasomaalisen hajotuksen kautta. Isoformi A:n, mutta ei B:n, yliekspressio näytti myös laskevan endogeenisen ubikiliini-1:n proteiinitasoja SH-SY5Y-solulinjassa, mikä viittaisi siihen, että proteiini-isoformit voivat interaktoida eri proteiinien kanssa eri tavoin. Ihmisen aivonäytteissä ei havaittu korrelaatiota patologisen toistojaksomonistuman ja C9orf72:n tai muiden tutkittujen FTD:aan liittyvien proteiinien tasojen välillä. Havaitsimme kuitenkin isoformi B:n proteiinitasoissa pientä laskua, mutta tulos ei ollut tilastollisesti merkittävä ja se täytyy varmentaa käyttämällä vielä toista vasta-ainetta. Tämä pro gradu –työ on tuottanut uutta tietoa C9orf72-proteiinin säätelystä ja vuorovaikutuksista sekä *C9ORF72*:n patologisen heksanukleotiditoistojaksomonistuman vaikutuksista, vaikka lisää tutkimuksia tarvitaankin jatkossa selvittämään *C9ORF72*:n fysiologisia ja patofysiologisia vaikutuksia.

FOREWORD

I have conducted the experimental part of my Master's thesis in Summers 2013 and 2014 and the writing during Autumn 2014 to Spring 2015. The possibility to complete my Master's thesis in the Neurobiology of neurodegenerative diseases research group in the Institute of Clinical Medicine - Neurology has been an exciting and educating experience which has given me insight into the fascinating and complex world of research and science overall. This experience has further assured me that delving into the wide field of research is the right path for me in the future. Especially I want to thank my supervisors PhD,Associate Professor Annakaisa Haapasalo and MSc Mari Takalo for giving me this opportunity and guiding me patiently through arising practical issues and writing. I'm also grateful to MSc Mikael Marttinen for his help with visual part of the thesis and further to the whole research group for all the practical help during these few years. I hope that I can continue learning under your assistance and work with you in the future.

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Stina Leskelä

ABBREVIATIONS

AD	Alzheimer's disease
ADARB2	Double-stranded RNA-specific editase B2
ALS	Amyotrophic lateral sclerosis
ASO	Antisense oligonucleotide
BSA	Bovine serum albumin
C9FTD/ALS	Concomitant FTD/ALS caused by *C9ORF72* gene mutation
C9ORF72	Chromosome 9 open reading frame 72 gene
C9orf72	C9orf72 protein coded by *C9ORF72* gene
CNS	Central nervous system
DENN	Differentially expressed in normal and neoplastic cells domain
DPR	Dipeptide repeat protein
FMR	Fragile-X mental retardation
FRDA	Friedreich's ataxia
FTD	Frontotemporal dementia
FTLD	Frontotemporal lobar degeneration
FUS	Fused in sarcoma RNA-binding protein
FXTAS	Fragile-X tremor/ataxia syndrome
GAPDH	Glyseraldehyde-3-phosphatedehydrogenase
GRN	Granulin gene
HD	Huntington's disease

hnRNP	Heterogenous nuclear ribonucleoprotein
iPSC	Induced pluripotent stem cell
MAPT	Microtubule-associated protein tau gene
p62/SQSTMI	p62/sequestosome protein
PLIC	Ubiquilin protein
Purα	Transcriptional activator protein Pur-alpha
RAN	Repeat associated non ATG-translation
RP-PCR	Repeat-primed PCR
SCA	Spinocerebellar ataxia
SH-SY5Y	Neuroblastoma cell line originating from human
TBST	Tris-buffered saline
TDP-43	Tar DNA-binding protein 43
UPS	Ubiquitin-proteasome system

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1 INTRODUCTION

Population studies indicate that neurodegenerative diseases are the third most common long-term diseases affecting the elderly. The number of demented patients is increasing globally, which is partly explained by the growing number of elderly persons in the population nowadays. Alzheimer's disease (AD) is the most common neurodegenerative disease causing dementia in the elderly but in this research project, we have concentrated on the second most common cause of dementia within the working-age population. This is frontotemporal dementia (FTD), the most common type of frontotemporal lobar degeneration (FTLD), which causes atrophy in the frontal and temporal lobes of the brain. The most common symptoms of FTD are changes in personality, difficulties to speak and loss of the meanings of words (1). Mutations in several genes, such as microtubule-associated protein tau (*MAPT*) or granulin (*GRN*), have been identified in patients with FTD (2, 3). Recently, the major genetic cause behind this disease was found to be a mutation in the gene *C9ORF72* i.e. chromosome 9 open reading frame 72 (4, 5), which leads to a hexanucleotide repeat expansion in the noncoding region of the gene. Since the mutation is particularly common in the Finnish population and has been estimated to be the cause of the disease in almost 50 % of the patients suffering from familial FTD and in 30 % of all FTD cases in Finland (6, 7), it is interesting and important to study this disease and the newly found gene behind it in greater detail.

FTD is divided into two types depending on the etiology of the disease. If a patient has a relative who also suffers from a form of FTD, which is caused by a same gene mutation, the mutated gene may be inherited and thus this type of disease is called familial FTD. Sporadic cases, on the other hand, have no clear genetic background, so they are believed to have arisen for the first time in that particular patient (8). Clinically FTD cases are divided into three groups: behavioral variant, progressive nonfluent aphasia and semantic dementia (1). Interestingly, FTD shares common symptoms and pathological mechanisms with the motor neuron disease amyotrophic lateral sclerosis (ALS). Some patients with FTD may suffer from symptoms that are regarded to be typical symptoms for ALS, such as muscle weakness and difficulties to breathe and swallow. On the other hand, patients with ALS can suffer from cognitive impairment (9, 10). These cases are classified as concomitant FTD/ALS and often the mutation in *C9ORF72* is the linking genetic factor behind these cases (C9FTD/ALS).

The mutation in *C9ORF72* leads to a GGGGCC hexanucleotide repeat expansion in the intron between the first two exons of the gene (**Figure 1**). This gene is shown to produce three different

mRNA variants from the genomic DNA. Two of these transcript variants, TV1 and TV3, produce the so-called long protein isoform A, which is a 481 amino acid-long protein consisting of exons 2-11. Transcript variant TV2 produces the short protein isoform B, which is 222 amino acids-long and consists of exons 2-5 (4). In patients with the hexanucleotide repeat expansion, it seems that levels of all variants are decreased possibly through promoter and histone hypermethylation (4, 11, 12, 13, 14, 15). It is believed that the decreased levels of mRNA lead to haploinsufficiency, in which the levels of C9orf72 protein are reduced and thus the normal cellular function of the protein is expected to be at least partially lost (4). Another possible mechanism leading to FTD has been suggested to be toxic gain of function, in which the expanded repeat sequesters RNA-binding proteins in the nucleus or alternatively produces through repeat associated non-ATG translation (RAN-translation) dipeptide repeat proteins (DRP), which accumulate into brain tissue.

The normal physiological functions of the protein isoforms A or B are still largely unknown, but bioinformatic analyses have identified similar structures between these proteins and so-called DENN (differentially expressed in normal and neoplastic cells) proteins (16). DENN-proteins activate Rab-GTPases and these proteins are known to regulate membrane trafficking inside cells (17). In accordance with its potential function in membrane trafficking, a recent study provided experimental evidence that C9orf72 associates with endosomes and regulates endosomal trafficking (18).

FTD cases can be divided into different neuropathological subgroups depending on what kind of protein inclusions are found in the brain tissue of the patient. For example, these inclusions may include tau, TAR DNA-binding protein 43 (TDP-43), fused in sarcoma RNA-binding protein (FUS), or proteins from the ubiquitin-proteasome system (UPS) (19). Similar inclusions have not only been found in FTD and ALS cases, but also in other neurodegenerative diseases such as dementia with Lewy bodies and AD (20). However, one type of possibly toxic protein inclusion that appears to be specific for FTD, contains proteins formed by RAN-translation (15). This RAN-translation produces dipeptide repeat-containing proteins from the GGGGCC hexanucleotide repeat, which are possibly toxic and accumulate in the cytoplasm of neuronal cells. Since these proteins are specifically found in patients who carry the pathological repeat expansion, these proteins could potentially be used as a biomarker for C9FTD/ALS (15, 21).

2 LITERATURE REVIEW

2.1 FTD/ALS

As mentioned earlier, in addition to the three clinical phenotypes of FTD, features of FTD can be seen in another disease called ALS. ALS is a motor neuron disease characterized by selective loss of either upper or lower motor neurons in brain and spinal cord. Upper motor neurons are in charge of signaling in cortex whereas lower motor neurons transport electrical impulses from brain to muscles (22). Degeneration of either upper of lower motor neurons causes muscle weakness, paralysis and ultimately death, usually caused by respiratory failure three to five years after symptoms have occurred.

The clinical overlap between ALS and FTD has been long known. Genetic studies had previously shown that both of these diseases were linked to the short arm of chromosome 9. Later, the genetic association was found to be linked to locus 9p21 but no amino acid-changing mutation was found from that region. Based on that it was believed that the genetic mutation was leading to FTD pathogenesis either by altering RNA splicing or gene expression, and that the mutation was located in a 232 kb intronic area (6, 23). In 2011, the common genetic cause in these diseases was found to be a hexanucleotide repeat expansion in a gene called *C9ORF72* (4, 5). Lomen-Hoerth and colleagues (2002) stated that 14 % of FTD patients also fulfill the clinical criteria of ALS and 36 % of FTD patients present symptoms that are regarded characteristic of ALS (9). On the other way around, approximately 50 % of ALS patients suffer from dementia and have cognitive impairment caused by atrophy in frontal and temporal lobes of the brains (10). Because of the significant overlap of FTD and ALS, the continuum of these two diseases is nowadays called FTD/ALS and if the *C9ORF72* repeat expansion is the genetic defect behind the disease, the acronym C9FTD/ALS is used. Still, it remains unknown which factors and molecular pathways determine if a *C9ORF72* mutation carrier develops FTD, ALS or the combined FTD/ALS. It is believed that a complex interplay between many genetic and environmental factors contribute to disease onset, development and clinical characteristics.

2.2 *C9ORF72* gene expression and function

The gene *C9ORF72*, located in chromosome 9, produces three different transcript variants by alternative splicing. Two of these transcript variants, TV1 and TV3, produce the so-called long protein isoform A which is a 481 amino acid long protein consisting of exons 2-11. Transcript variant TV2 produces the short protein isoform B, which is 222 amino acids long and consists of exons 2-5 (4). The *C9ORF72* gene structure, location of the hexanucleotide repeat expansion and the three transcript variants are depicted in **Figure 1**.

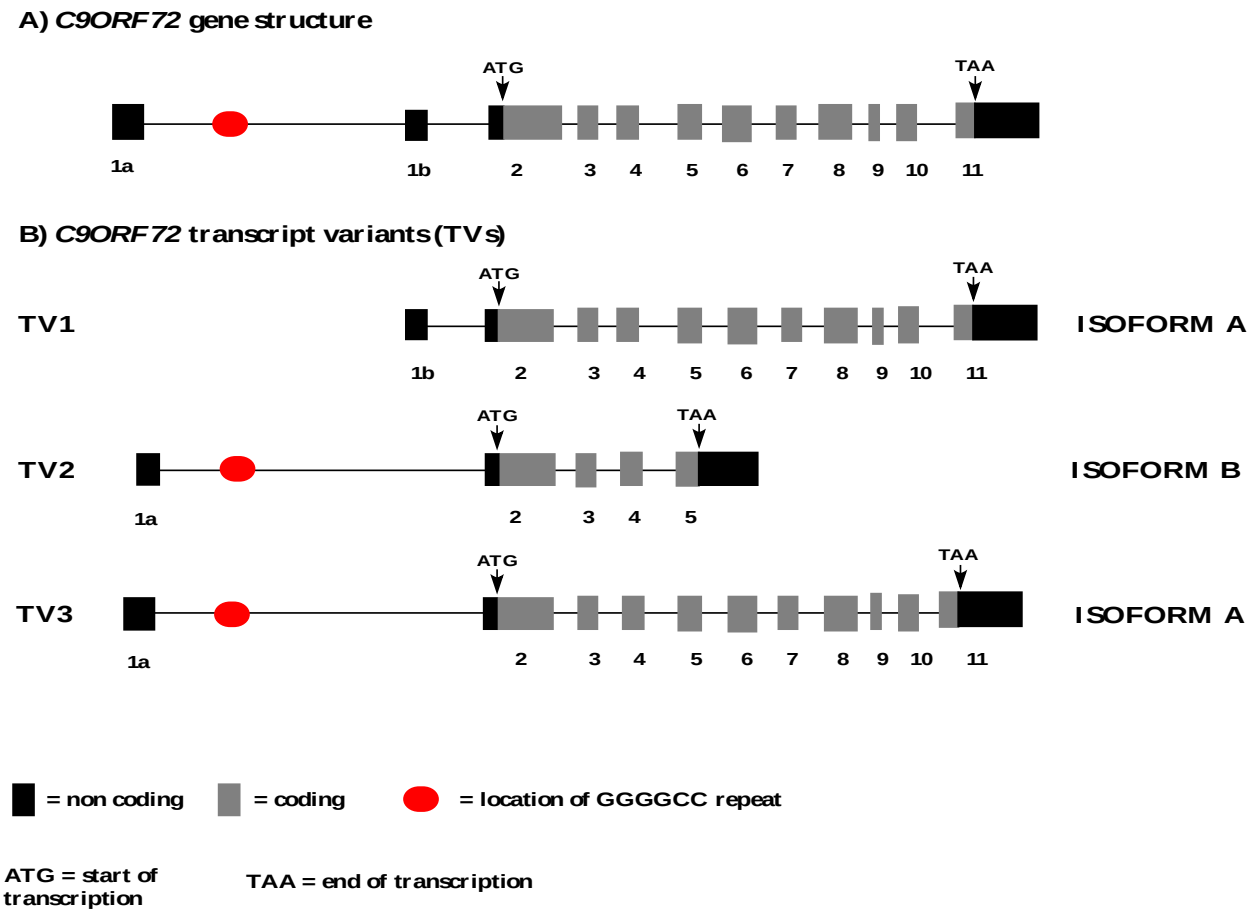

Figure 1: The structure of *C9ORF72* gene, transcript variants (TVs) and location of the hexanucleotide repeat expansion. A) The genomic DNA which encodes three different transcript variants. Black boxes indicate non-coding and gray boxes coding regions, respectively. **B)** Transcript variants 1 and 3 encode protein isoform A and transcript variant 2 protein isoform B.

In humans, all the transcript variants are expressed in the brain and in other tissues. The level of expression varies; the long protein isoform A is highly expressed in the brain and testicles (4, 21). Overall *C9ORF72* gene expression is reported to be the highest in the central nervous system (CNS) where it has been detected in brain areas such as cerebellum, frontal cortex, hippocampus, hypothalamus and spinal cord (5). Based on gene expression analysis, almost 93 % of all *C9ORF72* mRNA were transcript variant 1. This is also confirmed in other studies (12, 24, 25). These studies have reported an increase in TV2 and TV3, (which contain exon 1a i.e. GGGGCC repeat), and a decrease in TV1 levels in cell lines carrying *C9ORF72* repeat mutation when compared to controls without mutation. In addition to cell lines, the reduction in TV1 is observed also in expansion carriers, where the expression has been shown to reduce approximately 50 % from the normal level (4, 11, 13, 24, 25). The production of TV2 and TV3 require transcription through expanded GGGGCC repeat and thus it is speculated that this mutation is associated with the increase in TV2 and TV3 levels, possibly through stabilization of GGGGCC containing mRNA (15, 24). In accordance to reduced TV1 mRNA levels found in expansion carriers, reduction has also been observed at the protein level, where lower isoform A protein levels were detected in the frontal cortex but not in cerebellum. Both tissues seemed to have significantly lower levels of protein when only exon 1b-containing protein levels, i.e. proteins translated from TV1, were measured. This is consistent with the finding of reduced TV1 mRNA expression levels (25). Sareen and colleagues suggest that overall *C9ORF72* expression is not decreased and the observed loss of TV1, seen in other studies, is replaced by increased production of TV2 and 3, which contain the hexanucleotide repeat sequence (24, 26). Nevertheless, further studies are needed to evaluate whether this observation holds true. Methylation of the promoter region of *C9ORF72* has been shown to reduce the levels of all transcript variants, but demethylation seemed to activate only production of TV2 and 3 and not TV1 (12). Methylation has also been observed in the repeat expansion itself since the GGGGCC provides an additional CpG island where methylation can occur (27). Methylation in the repeat expansion itself seemed to be more common in larger repeat sizes compared to smaller ones.

The normal function and the cellular location of C9orf72 protein remained unknown until recently. De-Jesus Hernandez and colleagues (2011) reported that this protein locates mainly in cytoplasm whereas Renton and colleagues (2011) showed that the protein was found mostly in the nucleus of human fibroblasts and motor neurons of mice (4, 5). A more recent study has elucidated that the C9orf72 proteins are found in both the nucleus and cytoplasm of neuronal cell lines. In SH-SY5Y cell line, the long isoform A seems to localize more abundantly in the nucleus, whereas both isoforms

were detected in the cytoplasm (18). The function of C9orf72 proteins has been studied by searching homologous structures and amino acid sequences with other proteins with a known function. These bioinformatic studies have elucidated that C9orf72 proteins have similar structures with DENN proteins (16, 28). DENN proteins are well conserved proteins regulating the activation and inactivation of Rab-GTPases by adding guanosine triphosphate (GTP) or guanosine diphosphate (GDP) to these proteins. The Rab-GTPase family is known to have a role in regulation of membrane trafficking inside cells (17). Functions of individual Rab-GTPases are depicted in **Figure 2**. For example Rab1 protein regulates the membrane trafficking from endoplasmic reticulum to Golgi apparatus and Rab2 vice versa. Other functions that are regulated by Rab-GTPases are membrane trafficking from trans-Golgi to cell membrane, exocytosis of vesicles and guiding of vesicles to lysosomal degradation and recycling (17, 29). The homologous domains found in both C9orf72 and DENN proteins strongly suggest that these proteins have a role in membrane trafficking. To confirm this theory, a recent study reported a coincidence of C9orf72 proteins with endosomes in neuronal cell lines and human spinal cord motor neurons. C9orf72 seemed to colocalize with Rab5, Rab7 and Rab11 in mouse primary cortical neurons and motor neurons from human spinal cord, implying a role for C9orf72 proteins in endocytosis and autophagy in neuronal cell lines (18). If C9orf72 proteins function as DENN proteins and activate Rab-GTPases, the loss of function might lead to dysfunctional intracellular trafficking, protein degradation and recycling (28). Axonal transport defects are common in neurodegenerative diseases and defects in transport systems could underlie behind disease pathology of many neurodegenerative diseases (30).

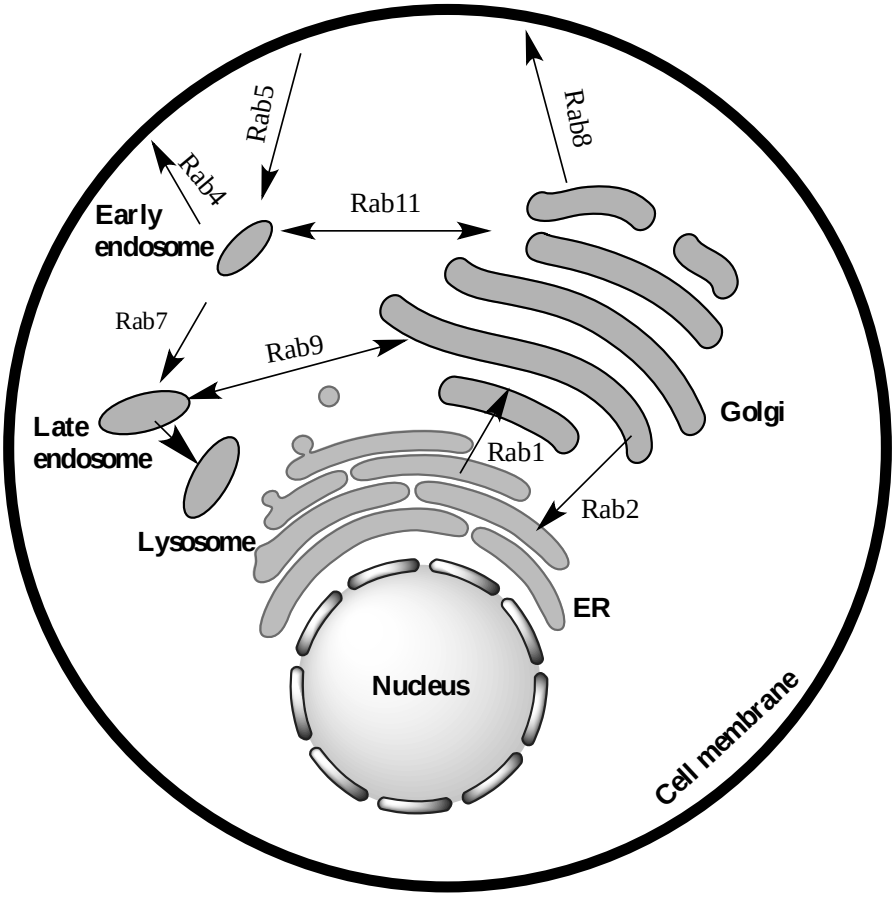

Figure 2: The function of selected Rab-GTPase proteins. The Rab-GTPase protein family, consisting of approximately 70 types of Rab proteins, has a known function in membrane trafficking, vesicle transport and protein degradation. Trafficking steps regulated by specific Rabs are indicated as arrows. C9orf72 protein products are believed to activate these Rab-GTPases and thus regulate membrane trafficking. Abbreviation: ER endoplasmic reticulum.

2.3 *C9ORF72* hexanucleotide repeat expansion

In healthy persons the number of hexanucleotide repeat varies typically from 2 to 23 repeats. It has been reported that the number of these repeats in FTD/ALS patients can vary from 700 to even 1600 repeats (4). There seems to be no cut off value, which would cause FTD, ALS or the combined FTD/ALS, but it has been estimated that over 30 repeats is pathological (5). Studies comparing clinical characteristics of FTD, ALS and concomitant FTD/ALS patients carrying *C9ORF72* hexanucleotide mutations to those of non-carriers found a tendency towards an earlier onset and shorter disease duration in mutation carriers suggesting that the expanded allele is a prognostic marker for poor survival (11, 31). Incomplete penetrance of the *C9ORF72* mutation has been observed in

some studies, meaning that the expanded allele has been found in patients without any signs of dementia or other characteristic FTD/ALS symptoms (32).

In other repeat expansion diseases such as Huntington's disease (HD) and myotonic dystrophy, a phenomenon called anticipation is often observed (33, 34). This means that the age at onset is lower from one generation to the other and the symptoms exacerbate in every generation. The phenomenon is explained by instability of repeats which accumulate in cells over generations and cause abnormal cellular functions. For example, in Huntington's disease, the CAG repeat expansion has been noticed to increase and multiply between generations (34). Based on this, it could be assumed that the *C9ORF72* repeat expansion would behave in a similar manner, but solid proof of anticipation lacks from FTD families (5). Some studies have found that occasionally the age at onset is earlier in subsequent generations and thus further studies are needed in order to determine accurately whether anticipation is observed in patients carrying the *C9ORF72* repeat expansion or not (4, 11, 35). The instability of the repeat, high GC-content and variation of repeat number between different cells complicates these studies. Conventional PCR method used in detecting the number of repeats is inaccurate and gives faulty results especially, when the number of repeat expansion rises to several hundreds of repeats. Thus repeat primed-PCR (RP-PCR) is used nowadays in detection (4). Also the Southern blot method is inoperative since it produces smear in to the gel instead of accurate bands due to the variation in repeat number in different cells. To circumvent this problem, optimized Southern blot methods have been developed in order to verify the repeat number unambiguously (36). A recent study compared the concordance rate of different methods used in 14 clinics to verify repeat size and revealed that there was divergence and false positive and negative results when detection was made with only one method, such as RP-PCR. Thus, combination of amplicon-length analysis with RP-PCR is suggested to be the best method and to give most accurate results needed in clinics and research work (37).

Several different research groups have studied the frequency of the *C9ORF72* repeat expansion in different populations. These studies have shown that the repeat expansion is particularly common in the Finnish population; it has been estimated to be the cause of 19 % of sporadic FTD cases and even 48 % of familial FTD cases (7). In addition to Finnish population, the repeat expansion is common in French, Canadian and North American populations (38). Overall, this pathological mutation is estimated to be the cause of 5.8 % of sporadic FTD cases and 25 % of familial FTD cases worldwide (7). **Figures 3** and **4** show the frequencies of *C9ORF72* gene mutation in sporadic and familial FTD and ALS cases in the Finnish population and worldwide.

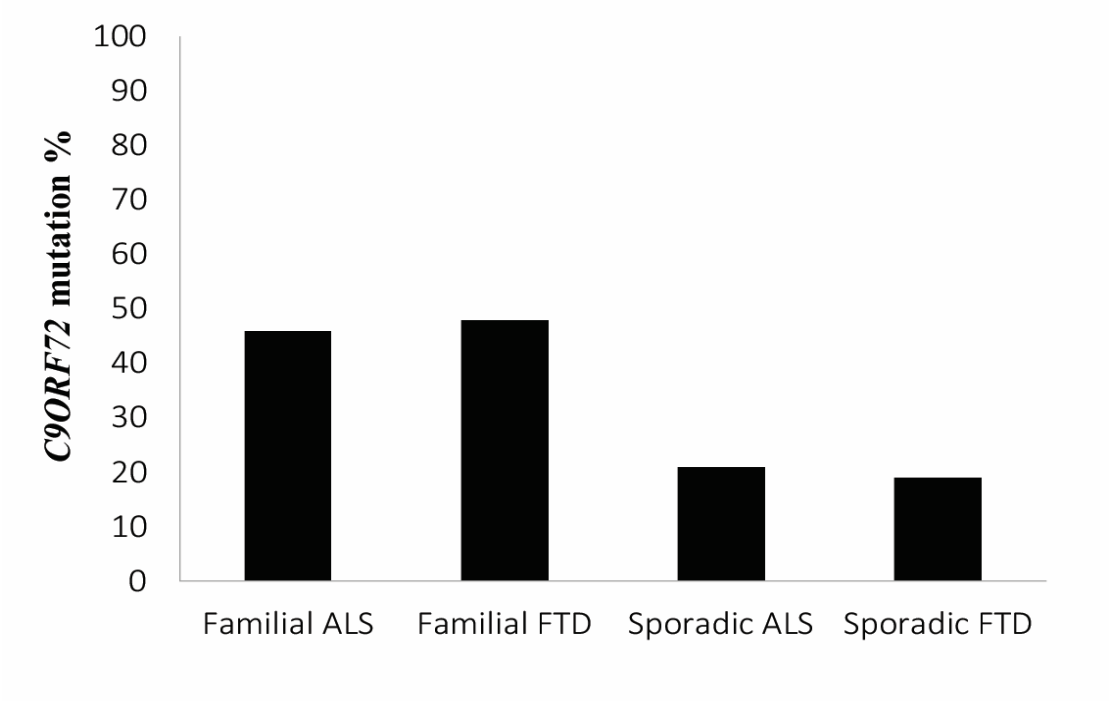

Figure 3: Frequency of *C9ORF72* mutation in the Finnish population. Diagram shows *C9ORF72* mutation frequency in familial and sporadic FTD and ALS cases. Notably, the mutation is found in almost half of familial FTD and ALS cases in the Finnish population. (Modified from van Blitterswijk et al. 2012).

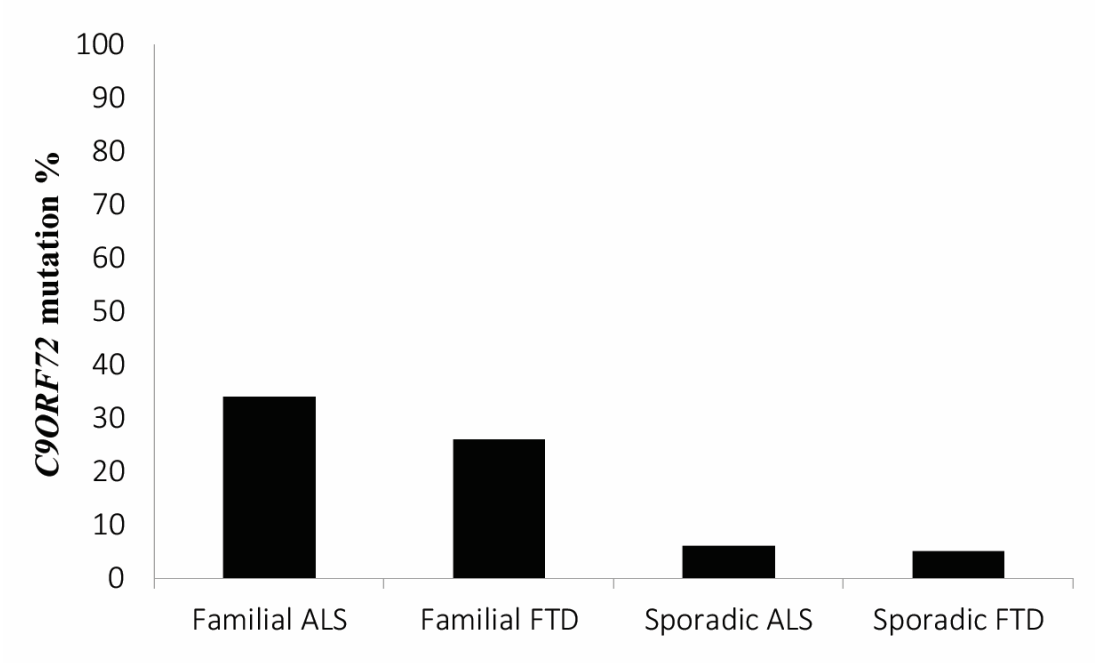

Figure 4: Frequency of *C9ORF72* mutation worldwide. Diagram shows that the *C9ORF72* mutation is the most common in patients with familial ALS and FTD. It has been detected in 34 % and 26 % of ALS and FTD patients, respectively. (Modified from van Blitterswijk et al. 2012)

2.4 Neuropathology of FTD

Atrophy in the frontal and temporal lobes of brain and gliosis are characteristic features of FTD. Gliosis is a phenomenon, in which the astrocytes supporting neuronal cells start to divide in damaged brain area and ultimately produce so-called glia scar. In addition, formation of vacuoles i.e. vacuolization has been detected in the cortex of patients diagnosed with FTD (1, 23). Different types of protein inclusions in either the cytoplasm or the nucleus of neuronal cells in various brain regions are also typical in FTD patients. These inclusions can be detected by immunohistochemistry and based on the type of the proteins in the inclusions, the disease is divided into five frontotemporal lobar degeneration (FTLD) subgroups which are: 1) FTLD-TAU, 2) FTLD-TDP, 3) FTLD-FUS, 4) FTLD-UPS and 5) FTLD-ni (39, 40). In FTLD-TAU, the inclusions found inside nerve and glia cells contain tau protein. In FTLD-TDP, inclusions have formed of TDP-43 and ubiquitin. These inclusions have been found in the cytoplasm and nucleus of nerve cells and are more common in patients without the *C9ORF72* mutation compared to those with the repeat expansion (11). The subtype which contains FUS protein is called FTLD-FUS. FTLD-UPS subtype inclusions contain proteins that can be detected with ubiquitin, ubiquilin and p62 antibodies but not with antibodies against FUS or TDP-43. In *C9ORF72* mutation carriers, these inclusions seem to be more common in distinct neuroanatomical regions compared to TDP-43 inclusions (11, 31, 39, 41). The last subtype is named FTLD-ni, in which distinctive inclusions have not been found (20, 40). To discover the underlying mechanisms behind the onset and development of FTD/ALS in *C9ORF72* mutation carriers, attempts to develop potential animal and cell culture models to study this gene and its role in FTD/ALS pathogenesis have been pursued. These models consist of induced pluripotent stem cells (iPSC), which have been induced to form neurons, genetically modified cells, or animal models which either overexpress GGGGCC repeats or in which *C9ORF72* orthologous genes are downregulated.

2.4.1 *C9ORF72* loss of function theory

In *C9ORF72* mutation carriers, it has been suggested that loss of function is one plausible mechanism leading to cellular aberrations and cell death (**Figure 5**). This theory is supported by the fact that a similar pathogenic mechanism is found in other repeat expansion diseases, such as in Fragile X mental retardation (FMR) and Friedreich's ataxia (FRDA) (42, 43). Both FTD- and ALS-related gene mutations and proteins associated to these diseases have been studied in organisms such

as *Caenorhabditis elegans*, *Drosophila melanogaster*, *Danio rerio* and *Mus musculus* (13, 44, 45, 46). From these models, orthologous genes for *C9ORF72* have been found in *C. elegans D. rerio* and *M. musculus* and thus these models are ideal for studying the knockdown effects of the normal C9orf72 protein. The expansion in *C9ORF72* gene has been mostly studied in non-vertebrates *D .melanogaster* and *D. rerio*. Studies in *D. rerio* have shown that knockdown of endogenous *C9ORF72* leads to abnormalities in neuronal development and changes in normal behavior of these fish (13). For example the knockdown animals had disrupted arborization and shortened motor neuron axons compared to controls. This phenotype was rescued with human *C9ORF72* mRNA, providing proof for a direct role of *C9ORF72* in disease phenotype. Similarly, researchers studying the effects of *C9ORF72* knockdown in *C. elegans* have reported that the human *C9ORF72* orthologous gene *alfa-1(ok3062)* null mutants showed disruptions in the motor phenotype, were more sensitive to osmotic stress and underwent neurodegeneration of GABAergic neurons (46). This *alfa-1(ok3062)* is reported to have 58 % homology with human *C9ORF72* and it seems to interact with TDP-43 and FUS proteins, which are linked to both FTD and ALS. Together, these data from animal models supports the loss of function theory for a possible pathological mechanism in FTD/ALS.

Further proofs for the loss of function theory are the decreased levels of *C9ORF72* mRNA variants found in C9FTD/ALS patients (4, 11, 13, 14, 15). The observed decrease could be explained by either DNA hypermethylation or histone methylation in the promoter region of the *C9ORF72* gene. Notably, recent studies have reported hypermethylation in the promoter region of *C9ORF72* with pathological repeat expansion cases (12, 47). DNA hypermethylation is a phenomenon, in which multiple methyl groups have been attached to the DNA strand in order to silence gene expression in that genomic region. Methylation is usually located in so-called CpG islands, which are DNA regions with a high GC-content, and this function is required for silencing of genomic regions (48). One study comparing the DNA hypermethylation status to the clinical characteristics of the disease between C9ALS cases and controls without the expanded allele found a correlation in CpG island hypermethylation and shorter duration of the disease (47). Attachment of methyl groups into histone tails has also been observed to silence the gene expression leading to reduced *C9ORF72* mRNA levels in mutation carriers (14). In another study, the role of DNA hypermethylation in patients carrying *C9ORF72* mutation with repressive histone methylation marks was investigated and it was found that only 10 % of these had methylation marks in DNA. This implies that histone methylation has a greater impact on gene silencing than DNA hypermethylation (49). Overall, these results suggest that loss of function in *C9ORF72*, plays a role in observed cellular abnormalities and pathogenesis.

2.4.2 *C9ORF72* gain of toxic function theory

Another theory for initiation of pathogenesis in C9FTD/ALS cases is believed to be gain of function rather than the previously mentioned loss of function (**Figure 5**). This theory is supported by pathologic mechanisms found in other repeat expansion diseases, such as Myotonic dystrophy , Fragile-X tremor/ataxia syndrome (FXTAS), HD and different types of Spinocerebellar ataxias (SCA) (33, 34, 43, 50, 51). Toxic gain of function is believed to have a role in disease onset in *C9ORF72* repeat expansion carriers through accumulation of dipeptide repeat (DPR) protein inclusions (see 2.5.3) and formation of RNA foci. Formation of DPR proteins occur through unconventional RAN-translation which produces five different, possibly toxic, polypeptides which accumulate in neuronal cells. In RNA-foci formation the abnormally expanded repeat sequence sequestrates RNA-binding proteins, such as FUS, double-stranded RNA-specific editase B2 (ADARB2), heterogenous nuclear ribonucleoprotein A1, A3 and A2B1 (hnRNP A1, hnRNP A3, hnRNP A2B1), and transcriptional activator protein Pur-alpha (Purα) (18, 24, 26, 45, 52, 53). Together these complexes form so called RNA foci into nucleus of cells in cerebral cortex and spinal cord of patients (4). While TDP-43 inclusions are characteristic of C9FTD/ALS patients, there is no evidence that TDP-43 binds to the expanded repeat. However, it is experimentally shown to bind to hnRNPs (26, 54). This could explain why TDP-43 inclusions are characteristically seen in specific brain regions of C9FTD/ALS patients. Sequestration of RNA-binding proteins into the expanded repeat leads to the situation where these proteins are unable to perform their normal functions such as pre-mRNA splicing, causing cellular aberrations and possibly cell death (55). Studies elucidating the role of depletion of RNA-binding proteins in cell viability have shown that sequestration of these proteins into the expanded allele or knockdown of these genes decreases cells' tolerance to stress factors leading to reduced viability (52, 56). These results imply that sequestration of RNA binding proteins has a possible role in C9FTD/ALS pathogenesis (52). It remains unknown whether the RNA foci-mediated alterations in RNA processing and apoptosis are direct or whether they are only a secondary to some other pathological mechanism.

Even though multiple studies have reported that reduction in *C9ORF72* transcript levels causes pathophysiological alterations, as described in section 2.4.1, conflicting results have challenged the loss of function theory as a pathogenic mechanism in C9FTD/ALS. For example, reduction in *C9ORF72* levels in mammals is well tolerated. A study with mice showed that 60-70 % reduction in *C9ORF72* levels did not lead to any alterations in behavior or accumulation of cellular inclusions

characteristic to FTD/ALS (57). Consistent with this, iPSC-derived motor neurons from C9ALS patients seemed to tolerate knockdown of *C9ORF72* levels to 10 % when transcripts were guided to degradation with antisense oligonucleotides (ASOs) (24). Furthermore, knockdown of *C9ORF72* did not seem to cause differences in the survival of rat primary motor neurons compared to control cells. In addition, homozygous mutation in *C9ORF72* did not appear to cause a more severe phenotype compared to patients with one mutated allele, as would be expected if loss of function theory would be the cause of disease onset and development (58). On the contrary, overexpression of the hexanucleotide repeat expansion has been proven to be toxic in both cell cultures and animal models such as flies and zebrafish (26, 45, 53). Taken together, it seems that loss of function may not be sufficient to cause disease onset and phenotypic alterations, but it might co-operate with gain of function mechanisms.

Supporting the gain of toxic function theory, a recent study reported a longer survival time in C9ALS patients with higher methylation density in the promoter region compared to patients with lower methylation density (59). Methylation of GGGGCC –region has been shown to silence the gene expression and thus reduce the burden of RNA foci and DPR formation (12). Promoter methylation seemed to increase the tolerance to stress factors, and decrease the formation of RNA foci and DPR protein inclusions (12). These findings suggest that promoter methylation could have a protective effect within repeat expansion carriers (59). Genome-wide studies elucidating the transcriptome between C9FTD/ALS patients and controls without the hexanucleotide repeat expansion revealed clear differences between these two groups (52, 57). Hierarchical clustering divided C9FTD/ALS patients and control samples into different groups and expression level analysis showed upregulation of 122 genes and downregulation of 34 genes in C9FTD/ALS samples, as compared to controls (57). Further studies have demonstrated that these alterations were not due to the loss of *C9ORF72* gene expression levels since knockdown of *C9ORF72* levels by 90 % by ASOs did not result in a similar changes in transcriptome, which was observed in C9FTD/ALS patients. Taken together, this mounting evidence suggests that the loss of function in *C9ORF72* might not play as significant role in C9FTD/ALS pathogenesis as it has been hypothesized before. It is probable that both loss of function and gain of toxic function in *C9ORF72* repeat expansion carriers play a role in pathogenesis.

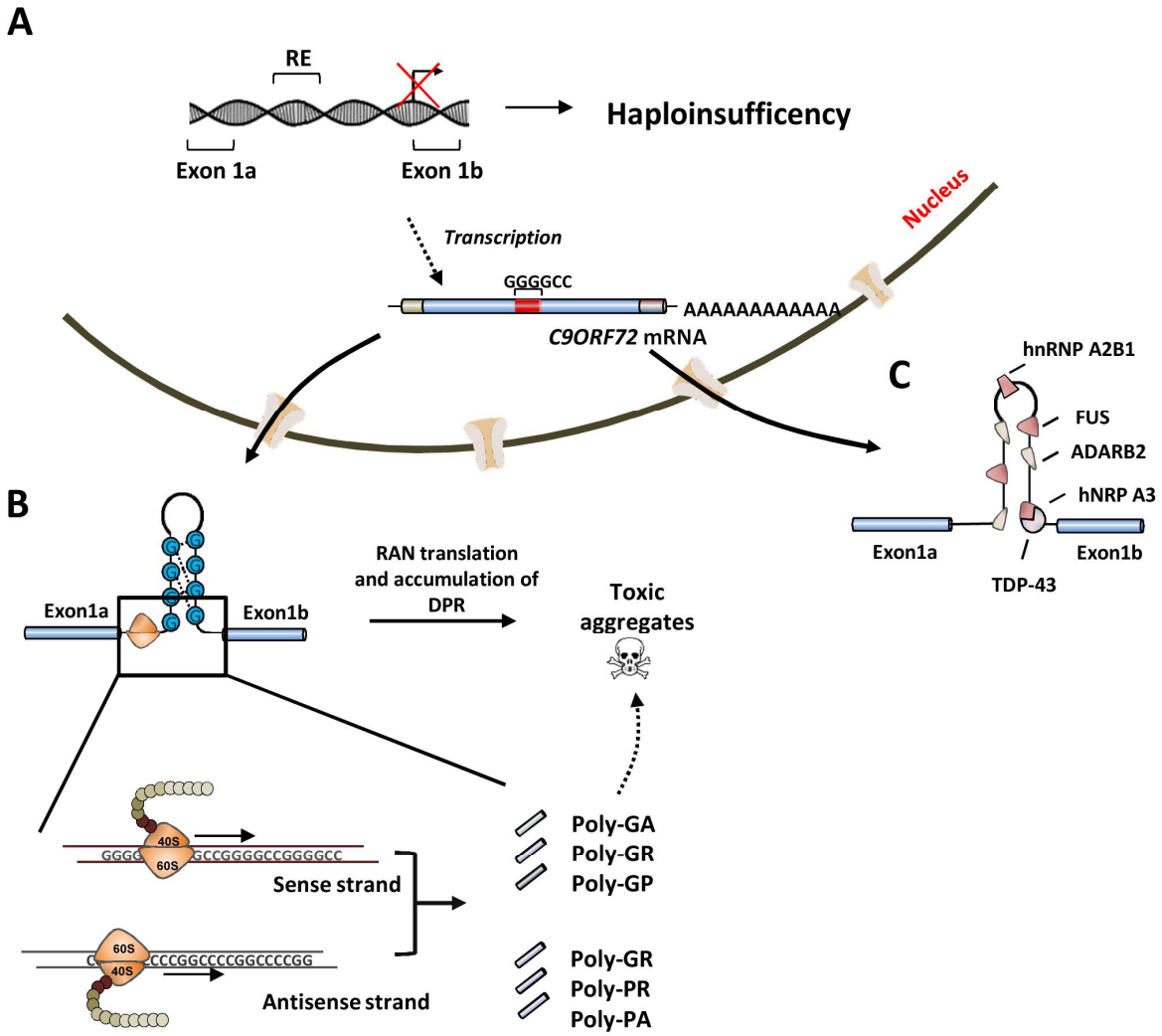

Figure 5: Candidate mechanisms leading to *C9ORF72* repeat expansion mediated toxicity. Three plausible molecular mechanism causing FTD/ALS development in GGGGCC hexanucleotide repeat expansion carriers. A) *C9ORF72* mutation has been proposed to lead to a situation called haploinsufficiency, where production of transcript variants are decreased. Reduction in C9orf72 proteins may lead to loss of function in membrane trafficking and protein recycling (18, 28). B) Formation of DPR proteins from expanded repeat sequence via RAN-translation. Production of five different DPR proteins from sense and antisense strands of expanded GGGGCC repeats causes toxicity and reduced cell viability in neurons (15, 21, 60, 61). C) Sequestration of RNA-binding proteins into RNA-foci. Repeat expansion has been shown to bind RNA-binding proteins such as FUS, Pur α, ADARB2 and family of hnRNPs. Sequestration of these proteins leads to alterations in RNA processing and thus possibly to reduction in cell viability (18, 24, 45, 52, 56).

2.5 Intracellular inclusions in FTD/ALS

2.5.1 TDP-43-positive inclusions

TAR DNA-binding protein 43 kDa (TDP-43) inclusions in the nucleus and cytoplasm of neuronal cells are characteristic in patients carrying the pathological *C9ORF72* repeat expansion. Overall, approximately half of FTD patients have inclusions consisting of TDP-43 in neuronal cells and glial cells. However, it appears that in C9FTD/ALS cases, the frequency of these inclusions is lower compared to other FTD subtypes (11, 31, 62). TDP-43-containing inclusions, and inclusions consisting of FUS proteins, are not only found in FTD patients but also in patients with other neurodegenerative diseases, such as AD, dementia with Lewy bodies and patients with corticobasal degeneration (20). Commonly the molecular pathology of C9FTD/ALS patients is described to be the B-subtype of FTLD-TDP. This neuropathology is characterized by dense cytoplasmic TDP-43 protein inclusions and few dystrophic neurites (40). The normal function of TDP-43 is to regulate RNA translation, modifications and splicing. In abnormal situations the protein is translocated from the nucleus to the cytoplasm, which causes disruption in the normal functions of the protein. Moreover, it has been noted that the translocation into the cytoplasm predisposes to abnormal accumulation of TDP-43 and ultimately formation of protein inclusions. Other pathological events besides translocation of TDP-43 protein are hyperphosphorylation, ubiquitination and N-terminal truncation of TDP-43 (55, 62).

In neurodegenerative diseases, the TDP-43 protein inclusions are believed to arise during formation of stress granules. Stress granules are protein inclusions that form in the cytoplasm during cellular stress and contain RNA strands and RNA-binding proteins, such as FUS, TDP-43, hnRNP A1 and hnRNP A2/B1 (18, 53, 55). Factors which may trigger this phenomenon include changes in temperature, exposure to chemicals, deprivation of glucose, oxidative stress and proteasomal stress (18, 55). During stress, cells pursue to save energy by decreasing translation of mRNAs to polypeptides. Inhibition of translation is achieved by forming cytoplasmic inclusions that contain mRNA and RNA binding proteins. When stress is ceased, the protein inclusions can be disassembled back to functional mRNA and RNA binding proteins (55). Disruptions in disassembling these inclusions may lead to increased formation and accumulation of stable inclusions inside nervous cells. It remains unknown whether observed cellular damage is caused by excessive formation and inadequate disassembly of stress granules or disruptions in RNA processing inside nucleus. On the

other hand, it has already been shown that depletion of RNA-binding proteins decrease cell viability during stress (51, 55, 56). Recently, one study reported that human bone osteosarcoma epithelial and mouse motor neuron-like hybrid cell lines expressing the *C9ORF72* repeat expansion have reduced ability to form stress granules compared to control cells expressing the normal size allele. They speculated that sequestration of RNA-binding proteins to the expanded repeat sequence impedes stress granule formation, suggesting that at least some pathological alterations in *C9ORF72* mutation carriers might be due to loss of RNA-binding protein function (63). However, TDP-43 has not been detected to co-localize with the hexanucleotide repeat, but it is known that family members of hnRNPs can directly interact with TDP-43 (26, 54). In addition, overexpression of *C9ORF72* under proteasomal stress induced formation of *C9ORF72*-containing nuclear inclusions and cytoplasmic stress granules (18). However, more studies are needed before further conclusions about the relationship between the *C9ORF72* repeat expansion and stress granule formation can be made.

2.5.2 Ubiquitin-, ubiquilin- and p62-positive inclusions

In addition to the previously described TDP-43-and FUS-containing protein inclusions, accumulation of proteins of the UPS is commonly observed in many neurodegenerative diseases (64). Protein degradation occurs through two main alternative pathways; UPS and autophagy. In UPS-mediated protein degradation, ubiquitin is covalently attached to the lysine residues of the proteins, which need to be degraded. These ubiquitin-tagged proteins may be guided to proteasome by ubiquilin family proteins (PLIC). In autophagy, proteins are also ubiquitinated, but the protein guiding these tagged proteins to degradation is suggested to be p62 (SQSTMI) instead of ubiquilin (65). Later, autophagosomes fuse with lysosomes, and specific lysosomal enzymes degrade the proteins (64). Dysfunctions in both of these protein degradation systems can lead to insufficient protein degradation and thus accumulation of proteins inside cells. Disruptions in the functions of the protein degradation machineries are often observed in neurodegenerative diseases, although the role of these findings in the pathology of neurodegenerative diseases remains elusive (64).

Ubiquitin pathology has been a target of research within C9FTD/ALS cases in order to determine the spreading of the pathology i.e. in which brain regions these inclusions are formed, and how these inclusions contribute to the pathology of the disease (66). Kersaitis and colleagues (2006) investigated brain samples of FTD, ALS and concomitant FTD/ALS cases and found that ubiquitin-positive

inclusions were found abundantly in the dentate gyrus and frontal and temporal lobes of almost all patients (66). Other brain areas seemed to harbor distinctly fewer inclusions. Regardless of the high frequency of these inclusions in some brain areas, they are believed to be insignificant in the etiology and progression of the FTD/ALS although they may be an indicative of dysfunctional protein degradation (64). Whether the reason for inclusion formation is the loss of normal function at the UPS or autophagy or gain of toxic function caused by accumulation of protein aggregates, which overloads the degradation machineries, remains unclear.

Ubiquilin is a protein family which functions as a shuttle between ubiquitinated proteins and the proteasome (67). Ubiquilin pathology is commonly found in ALS- and FTLD-TDP patients who carry the pathological repeat expansion and it has been estimated to be even more common than TDP-43 pathology in these cases (31). Ubiquilin pathology seems to be specific to C9FTD/ALS cases, since pathologists have been able to detect mutation carriers accurately from non-carriers based only on ubiquilin pathology. Further histological studies reporting that the mutation carriers harbor a more widespread and abundant ubiquilin pathology in the brain compared to non-carriers suggest that ubiquilin pathology is specific to *C9ORF72* mutation carriers. Brettschneider and colleagues (2006) state that the intracellular ubiquilin-positive inclusions found in the dentate gyrus are more common in ALS- and FTLD-TDP patients with the repeat expansion compared to cases without it. Also, the half-moon shaped ubiquilin-positive inclusions found in the cerebellum were only present in cases with the repeat expansion. These results suggest that ubiquilin pathology may play a role in repeat expansion-associated neurodegeneration. (31)

In addition to the previously described inclusions, it has been noticed that in C9FTD/ALS cases the protein inclusions often contain p62 protein (41). Based on the theory by Komatsu and colleagues (2007), disruptions in protein degradation machineries lead to formation of ubiquitinated p62 protein inclusions in cells (68). Accordingly, p62-and ubiquitin-positive inclusions were detected in the cortex of autophagy-deficient mice. On the other hand, Arai and colleagues (2003) and others have found p62 protein in ubiquitin-negative inclusions in glial cells of FTD patients with ALS (41, 69). Based on these results, it can be noted that p62 protein can be present in both ubiquitin-positive and-negative inclusions, but the contribution of these inclusions to the disease pathogenesis of disease is still unknown (69). In conclusion, deficiencies in protein degradation machineries may not be sufficient to cause neurodegeneration, but they implicate that alterations in proteins degradation is characteristic to neurodegenerative diseases.

2.5.3 Inclusions formed by RAN-translation

Another plausible mechanism underlying the pathology of C9FTD/ALS has been hypothesized to be related to RNA function, namely RAN-translation. This RAN-translation causes deviant peptide formation in FTD cases and in other types of repeat expansion disorders, such as SCA8, myotonic dystrophy and FXTAS (70). This non-canonical RAN translation is a phenomenon, in which the expanded *C9ORF72* produces DPR protein chains abnormally without ATG codon. ATG codon marks a starting point for DNA transcription and subsequently AUG is the start codon for protein translation in mRNA. An ATG start codon has been found in the close proximity of the repeat expansion, but there are also multiple stop codons between the start codon and repeat expansion to terminate transcription (21). The mechanisms behind this non-canonical translation remained unknown until recently. Secondary structure predictions and experimental evidence imply that expanded repeats can form relatively strong and stable secondary structures in both RNA and DNA level. These are termed hairpins or G-quadruplexes, which could mitigate the start of translation (21, 26, 71). Reddy and colleagues (2013) suggest that the guanine content in the expanded repeat, RNA concentration, and the length of the repeat have an effect on the formation and stability of the structure. For example, when the length of the expanded repeat increases, the structure is more stable (21, 71). Haeusler and colleagues (2014) suggest that the expanded repeat containing DNA and RNA separately form G-quadruplexes and together structures called R-loops (26).

Recent studies state that RAN translation through expanded GGGGCC-repeat produces DPR which consist of either glycine and alanine (GA), glycine and proline (GP), or glycine and arginine (GR), depending on the reading frame in the sense strand. All these three different types of DPR proteins have been found in the brain samples of *C9ORF72* expansion carriers and these inclusions appear co-localize with p62-positive neuronal inclusions characteristic of C9FTD/ALS (15, 21). Ash and colleagues (2013) and others found that poly-GP-peptides were the most abundant polypeptides in the cytoplasm of neuronal cells and iPSC-derived motor neurons. Others suggest that poly-GA-peptides are the most common type of DPR proteins (12, 15, 21, 52). Moreover Mori and colleagues (2013) reported that in addition to sense strand, the expanded repeat can also be read in the opposite direction in antisense strand. This can produce dipeptide proteins from the CCCCGG repeat expansion, consisting of proline-alanine (PA), proline-glycine (PG) or proline-arginine (PR). From these five different dipeptide proteins, produced from sense and antisense strands, only poly-GA and

poly-GP proteins are hydrophobic and thus these proteins are believed to accumulate and form inclusions in cytoplasm (15, 72). In accordance to this, poly-GA-protein inclusions have been found in the cytoplasm and nucleus of various brain regions in patients carrying the pathological repeat expansion (15, 61). The shape of these inclusions varies; inclusions in the cytoplasm have been reported to be granular and occasionally star-shaped whereas inclusions in the nucleus are small and round-shaped. There seems to be conflicting data regarding which DPR are most abundant and toxic. This may be partly due to some research groups concentrating only to sense strand-derived DPR instead of both sense and antisense strand-derived peptides. Zhang and colleagues (2014) report that poly-GA-containing DPR proteins are toxic in neuronal cells and mouse primary cortical cultures, where poly-GAs increased caspase-3 activation, disrupted UPS function, and caused ER stress (61). A recent study elucidating the potential toxicity of DPR, found that only arginine-containing peptides were toxic to cells in cortical and motor neuron cultures (60). They found that poly-PR dipeptides reduced cortical neuron viability. Poly-PR-containing inclusions were toxic to human iPSC-derived neurons and expression of these repeats also led to neurodegeneration in the fly. Toxicity of poly-PR aggregates was believed to result from their abnormal localization to nucleus, which seemed to cause swelling of the nucleolus and stress granule formation (60). These results suggest that aggregation of poly-PR dipeptides causes neurodegeneration through alterations in RNA translation.

Researchers have noticed that DPR proteins formed from the repeat expansion are specifically found in the brain tissue of patients who carry *C9ORF72* repeat expansion. It appears that DPR proteins are absent from other tissues, the only exceptions were Sertoli cells in the testicles of patients carrying the GGGGCC repeat expansion (21). These dipeptide inclusions were found both in the cytoplasm and nucleus. It is still unknown whether these inclusions are linked to the formation of other protein inclusions, but it is believed that poly-GA proteins may be the most important component of inclusions found in the brains of FTLD-UPS subtype patients (15, 21). On the other hand poly-GA proteins have been found inside TDP-43 inclusions, which has raised the question whether there is a connection between RAN-translation and TDP-43 pathology. It is possible that RAN-translation could functions as a preceding and initiating factor in the formation of TDP-43 pathology (15). A recent study reported that poly-GA proteins localize into TDP-43-negative and ubiquitin/p62-positive inclusions found in brain tissue of C9FTD/ALS patients. DPR proteins were also shown to decrease cell viability and disrupt protein degradation pathways leading to accumulation of proteinaceous aggregates. Notably, poly-GR and poly-PR proteins were observed to be present in TDP-43-positive inclusions and possibly alter TDP-43 protein homeostasis. These data suggest that DPR proteins

cause dysfunction in the UPS system, leading to altered homeostasis of proteins, such as TDP-43 (72).

2.6 Potential biomarkers and therapies

With the technology of today, there are no accurate and efficient ways to diagnose and treat patients with FTD/ALS. Further studies are needed to understand the disease pathogenesis of FTD better at the cellular and molecular level. This may ultimately lead to the identification of novel biomarker candidates or potential therapeutic targets. Therefore accurate detection of the GGGGCC repeat size would be important for both clinical purposes as well as for research in FTD/ALS. The high GC-content, large size and instability of the repeat expansion makes it hard to detect it accurately. Nowadays the best method for identification of *C9ORF72* repeat expansion carriers is RP-PCR, combined with amplicon length analysis (37). It is suggested that, due to somatic instability of the repeat expansion in different tissues, detection of the repeat size from blood does not accurately predict the repeat size in the brain (73). To further highlight the difficulties in using the repeat size as a biomarker, the repeat size is reported to vary also between different brain regions, and it seems that there is no correlation between repeat expansion size in blood and clinical characteristics of FTD/ALS (73). The methylation profile seems to be more stable than the size of repeat expansion in *C9ORF72* gene itself. Thus it has been postulated that the methylation density in the promoter region of *C9ORF72* or histone methylation could be used as a biomarker for C9FTD/ALS (14, 59). Hypermethylation of the expanded repeat has been reported to be associated with repeat expansion and it is detectable in blood (47, 59). Xi and colleagues (2013) studied the correlation between hypermethylation and clinical characteristics of FTD/ALS and found a negative correlation between hypermethylation and disease duration in C9ALS cases, compared to controls without the expanded allele (47). This observation could be explained by the fact that hypermethylation is strongly associated with the expanded repeat and the repeat expansion itself has been reported to cause faster disease progression compared to disease cases without expanded allele (31, 41, 59). When the relationship between hypermethylation and disease duration within *C9ORF72* carriers was compared, a positive correlation was found in C9FTD cases (59). Thus further studies with large patient cohorts are needed to evaluate the possible diagnostic and prognostic value of *C9ORF72* promoter or histone methylation in C9FTD/ALS cases.

In order to develop new therapies for treating C9FTD/ALS patients, it is important to determine whether *C9ORF72* loss or gain of function plays a role in disease pathogenesis. Overexpression as a therapy to treat the hypothesized loss of function in *C9ORF72* mutation carriers could have deleterious effects, as the overexpression could result in increased pathological burden through increased accumulation of RNA foci and formation of DPR protein inclusions (12, 59). Based on mounting evidence, it seems that gain of toxic function might be the major molecular mechanism behind C9FTD/ALS pathogenesis. To inhibit or reverse formation of RNA foci and DPR proteins, therapies to increase *C9ORF72* methylation or to target *C9ORF72* mRNA for degradation, could be utilized. Methylation of *C9ORF72* promoter region has been shown to decrease total levels of *C9ORF72* mRNA, and formation of RNA foci and DPR protein inclusions (12). RNA foci and DPR protein inclusions could also be inhibited by targeting *C9ORF72* mRNA with antisense oligonucleotides (ASOs, **Figure 6**). ASOs have already been developed and tested for other repeat expansion diseases, such as myotonic dystrophy and HD (74, 75). ASOs are synthetically derived nucleotides with homologous sequences to specific mRNA strand. ASO binding leads to target mRNA degradation or inhibition of translation of deviant transcripts. Obstacles with stability and sensitivity of ASOs have been overcome by modifying the nucleotide structure. Deficient cellular uptake can hinder treatment efficacy. For example in myotonic dystrophy it has been challenging to deliver ASOs to all affected tissues. This problem might occur in case of C9FTD/ALS as well, since ASO treatment should access all affected brain regions. On the other hand, studies with mice have elucidated a long-term reduction in C9orf72 levels in various brain areas after ASO injection to lateral ventricle. This implies that at least in mice the distribution of ASOs is wide (57). ASO-mediated decrease in *C9ORF72* gene expression seemed to reduce the formation of RNA foci, but it did not restore the altered gene expression profile towards control-like state (as described in section 2.4.2). This phenomenon could be partly explained by the targeting of ASOs to only the sense strand, since the same study reported antisense-derived RNA foci formation in the brain samples from *C9ORF72* mutation carriers. As a conclusion, it is evident that ASOs targeting both sense-and antisense-derived transcripts should be developed in order to evaluate whether this treatment alleviates the *C9ORF72* mutation-mediated toxicity.

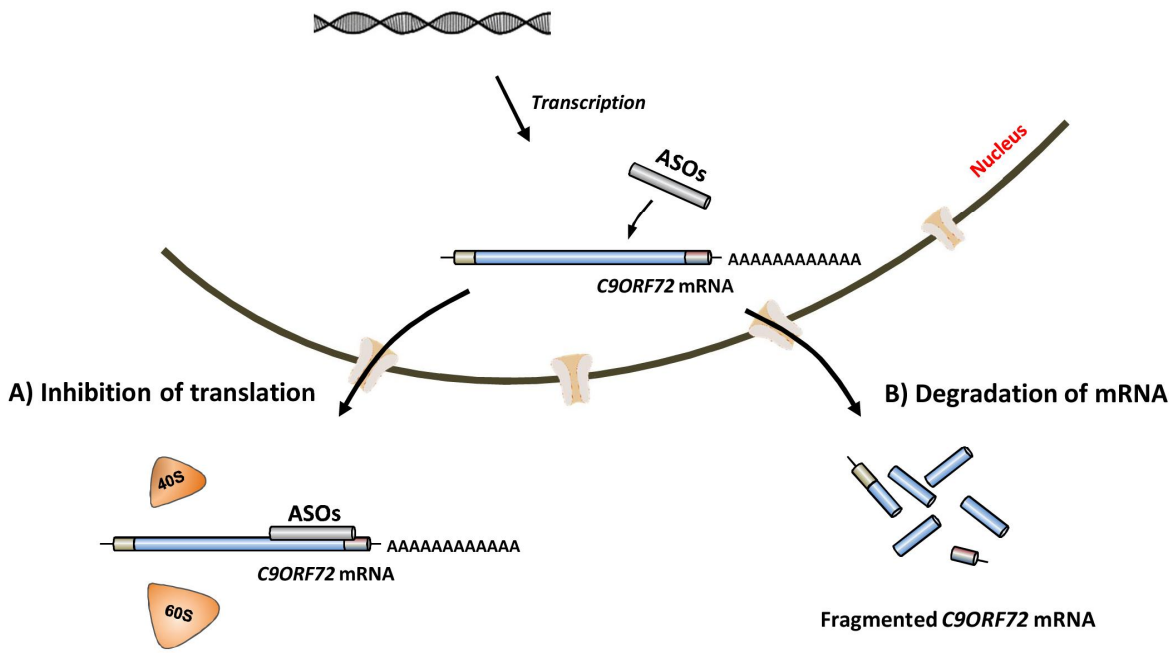

Figure 6: Mechanism of antisense oligonucleotides as a potential therapy in C9ORF72 repeat expansion carriers. ASO treatment could possibly be a new therapeutic method to treat patients with *C9ORF72* mutation. ASO treatment functions in two ways: A) Inhibition of *C9ORF72* mRNA translation and B) Degradation of *C9ORF72* mRNA.

Electrophysiological studies can be utilized to detect cellular alterations in the early phases of disease pathogenesis before any morphological or molecular signs of neurodegeneration can be observed. One study elucidating the excitability of C9ALS iPSC-derived motor neurons reported hyperexcitability in these neurons. Hyperexcitability was later followed by diminished synaptic activity and ability to form action potentials (76). This theory of hyperexcitability followed by hypoexcitability was supported by another study where C9ALS patient iPSC-derived motor neurons were observed to harbor decreased excitability (24). Notably, gene expression analysis showed decreased levels of transcripts, which were identified to have a role in membrane excitability, supporting the idea of hypoexcitability (24). Further, treatment with ASOs was reported to reduce the formation of RNA foci and lead to normalization of levels of certain differentially expressed genes. All in all, ample evidence from ASOs as a potential treatment for repeat-associated neurodegeneration in C9FTD/ALS cell and animal models suggest that this treatment is specific and effective to alleviate the *C9ORF72* mutation-mediated toxicity. Nevertheless, it must be noted that the experimental models should be validated well to increase the predictive value and to facilitate the successful

translation from animal models to clinical trials. Thus, further studies with animal models and patient iPSC-derived cells are needed before the ASOs could be targeted to clinical trials with patients.

3 AIMS

The main goal in this Master's thesis study was to elucidate the expression and function of *C9ORF72* gene in neuronal cells and human brain tissue samples. The functions of the individual C9orf72 protein isoforms A and B have not been extensively characterized in previous studies. The two isoforms differ in their lengths and isoform B lacks the protein sequence encoded by exons 6-11 present in the C-terminus of the isoform A. Therefore, it is possible that they display differential functions and protein interactions.

Overexpression of the isoforms A and B in SH-SY5Y human neuroblastoma cells was utilized to elucidate the expression and function of the C9orf72 protein isoforms and other FTD-associated proteins. In addition, we used proteasome inhibition to induce proteasomal stress, a condition involved in the pathogenesis of FTD and other neurodegenerative diseases, to examine whether the levels of C9orf72 or other FTD-related proteins were affected in SH-SY5Y cells overexpressing C9orf72 isoforms. Our hypothesis was that the proteasome inhibition will increase the levels and possibly cause aggregation of these proteins into insoluble inclusions in the cells. We also determined expression levels of the C9orf72 isoforms and other FTD-associated proteins in the brain tissue samples of patients with and without the *C9ORF72* hexanucleotide repeat expansion. Here, the aim was to investigate whether possible alterations in the levels of any of these proteins correlated with the presence of the repeat expansion.

4 MATERIALS AND METHODS

4.1 Cell culture, transfections, and induction of proteasomal stress

SH-SY5Y human neuroblastoma cells, derived from bone marrow, were cultured in Dulbecco's Modified Eagles Medium (DMEM) (Biowhittaker™, Lonza), supplemented with 10 % fetal bovine

serum (FBS), 2mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. During experiments, the cells were kept in an incubator (+37 ºC, 5 % CO_2) and divided when confluent.

For transfections, SH-SY5Y cells were harvested and plated at the density of 93750 cells/cm² on a 6-well plate. Next day, when cells had attached to plate surface, transient transfections were performed with cDNA constructs encoding C9orf72 isoform A or isoform B by using Lipofectamine 2000 reagent (LPF2000, Invitrogen) in Opti-MEM I reduced serum medium. Isoforms A and B were tagged with C-terminal GFP and Myc-DDK tags, respectively.

Next day, fresh medium was changed to all samples. The day after transfections proteasomal stress was induced by treating the cells with 10µM proteasome inhibitor lactacystin (Invitrogen) overnight (16-24h). Dimethyl sulfoxide (DMSO) was used as a control (Hybri-Max, Sigma).

The protein samples were collected from the transfected cells at 48 hours after transfection as described below.

4.2 Human brain tissue samples

Human postmortem frontal cortex samples were obtained from the Kuopio Brain Bank from patients diagnosed with FTD (3 samples), ALS (1 sample) or AD (6 samples). The *C9ORF72* hexanucleotide expansion status of these patients was previously determined at the Institute of Clinical Medicine – Neurology, UEF from blood DNA samples of the same patients by RP- PCR. The patients were classified as either positive (ORF+) or negative (ORF-) for the repeat expansion. Three of the FTD/ALS cases were ORF+ and one ORF-. All six AD patients were confirmed ORF-.

4.3 Protein extraction and Western blot analysis

Proteins were extracted from the transfected cells by scraping into Tissue Protein Extraction Buffer (T-Per), Thermo Scientific), supplemented with 1:100 protease and phosphatase inhibitors (Thermo Scientific). Cells were lysed on ice for 30 minutes. Total protein fraction was separated by centrifuging cell lysates for 10 min at 10 000g at +4°C. Protein concentrations were measured by using BCA kit (Pierce), based on so called biuret reaction. The absorbance correlates with protein

concentration and was measured with absorbance microplate reader (BioTek ELx808) at 562 nm wavelength. Bovine serum albumin (BSA) was used for creating a standard curve at specific concentrations.

Protein samples were prepared for gel run by adjusting protein amounts to 20-40 µg with T-Per supplemented with protease and phosphatase inhibitors. After that, 4x LDS loading buffer (Invitrogen) containing 20 % β-mercaptoethanol was added to samples. Protein samples were loaded to SDS-PAGE gel (NuPAGE Novex 4-12 % Bis-Tris mini, Life Technologies) and the samples were separated at 150 V. Proteins were blotted onto a polyvinylidenedifluoride (PVDF) membrane (Amersham Hybond-P) with TE77 ECL Semi dry transfer unit [NuPage Transfer buffer (Invitrogen) and 75 mA/transferred membrane].

After transfer, unspecific antibody binding was blocked with 5% non-fat dry milk in 1 x Tris-buffered saline-Tween-20 (TBST, 50 mM Tris, 150 nM NaCl, 0.1% Tween-20, pH 7.4) for 1 hour. Specific protein bands were detected by incubating the membrane with protein-specific primary antibodies overnight at +4°C and appropriate horse radish peroxidase-conjugated secondary (ECL™, anti-mouse or anti-rabbit IgG, GE Healthcare) antibodies for 1 hour at RT. All primary antibodies and dilutions used are listed in **Table 1**. The proteins were detected using ECL detection reagents (Amersham bioscience, GE Healthcare), where horse radish peroxidase enzyme produces chemiluminescence from luminol in presence of H_2O_2. Protein bands were imaged with ECL camera (GE Healthcare). The intensities of the detected protein bands were quantified with Quantity One (4.6.2) program (BioRad) and normalized to those of the house-keeping gene glyceraldehydetriphosphate dehydrogenase (GAPDH, loading control). The effects of transfections and treatment were assessed by comparing the protein band intensities of treated samples to control samples. The membrane was stripped from the old antibody with stripping buffer (Thermo Scientific) for 10 min at RT, after which it was blocked, incubated with another antibody and detected as described above.

Table 1: Primary and secondary antibodies used in Western blot detections.

Antibody	Type of antibody	Dilution	Molecular weight (kDa)	Producer
C9orf72	polyclonal rabbit	1:500	Isoform A 54 kDa Isoform B 25 kDa	Santa Cruz
FUS	polyclonal rabbit	1:1000	75	Sigma-Aldrich
GAPDH	monoclonal mouse	1:15 000	40	Abcam
TDP-43	polyclonal rabbit	1:1500	~ 45	Proteintech
Ubiquilin-1	polyclonal rabbit	1:1000	~ 62	Sigma-Aldrich

4.4 Statistical analyses

Protein band intensities detected with Western blot were normalized to those of GAPDH in each sample with Quantity One software. Results from cell culture experiments are shown as % (mean ± SEMs) of the levels in DMSO-treated pcDNA isoform A- or B-overexpressing or pcDNA samples (=100%). The data from brain samples are shown as relative intensity units of the protein bands normalized to GAPDH intensity and expressed as mean ± SEM. Calculation of statistical significance was performed with IBM SPSS software and determined with One-way ANOVA or Mann Whitney U test, where the threshold for statistically significant p-value was set to ≤ 0.05.

5 RESULTS

5.1 Effects of proteasome inhibition in *C9ORF72* overexpressing cells

In order to study the expression of C9orf72 isoforms, SH-SY5Y cells were transfected with cDNAs encoding isoform A or B tagged with GFP or myc-DDK, respectively. Proteasomal stress and resulting dysfunction of the UPS is a characteristic feature of various neurodegenerative diseases. Thus, we treated the cells with proteasome inhibitor lactacystin and analyzed whether overexpression of *C9ORF72* under proteasomal stress would lead to accumulation of C9orf72 isoforms. Quantification of GAPDH-normalized protein levels revealed that the proteasomal inhibition in SH-SY5Y neuroblastoma cells overexpressing C9orf72 isoforms A or B, significantly increased the levels of both isoforms (**p < 0.001 isoform A, ***p < 0.0001 isoform B; **Figure 7**). These results suggest that the degradation of C9orf72 isoforms is, at least partially, dependent on the UPS.

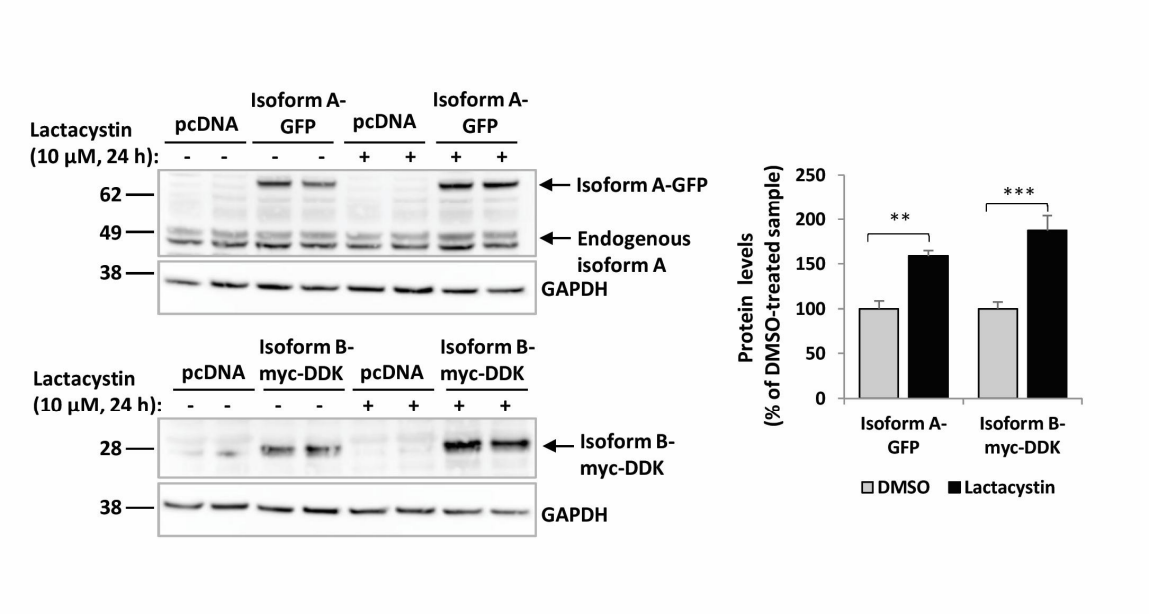

Figure 7. The levels of C9orf72 isoform A and B are significantly increased in SH-SY5Y cells in response to proteasomal inhibition. Western blot analysis of the proteins from the cells transfected with isoform A-GFP or B-myc-DDK and treated with the proteasomal inhibitor lactacystin (10 µM) or DMSO (vehicle) for 24 hours. Molecular weights are shown on the left of the Western blots as kDa. Quantification of the protein levels is shown on the right. The protein levels are normalized to those of GAPDH and shown as % of the levels in DMSO-treated samples (=100%). Mean ± SEM, n = 4. One-way ANOVA, **p < 0.001, ***p < 0.0001.

5.2 Endogenous ubiquilin-1 levels are decreased after overexpression of *C9ORF72* isoform **A**

To assess whether proteasomal inhibition in C9orf72 isoform A and B overexpressing SH-SY5Y cells would lead to accumulation of other FTD-related proteins, we analyzed the levels of ubiquilin-1, FUS and TDP-43 by western blot. Quantification of the endogenous levels of TDP-43, FUS and ubiquilin-1 are shown in **Figure 8**. The levels of endogenous ubiquilin-1 in DMSO treated C9orf72 isoform A overexpressing cells were reduced (*p < 0.05). Overexpression of C9orf72 isoforms during proteasome inhibition did not coincide with altered levels of TDP-43 or FUS.

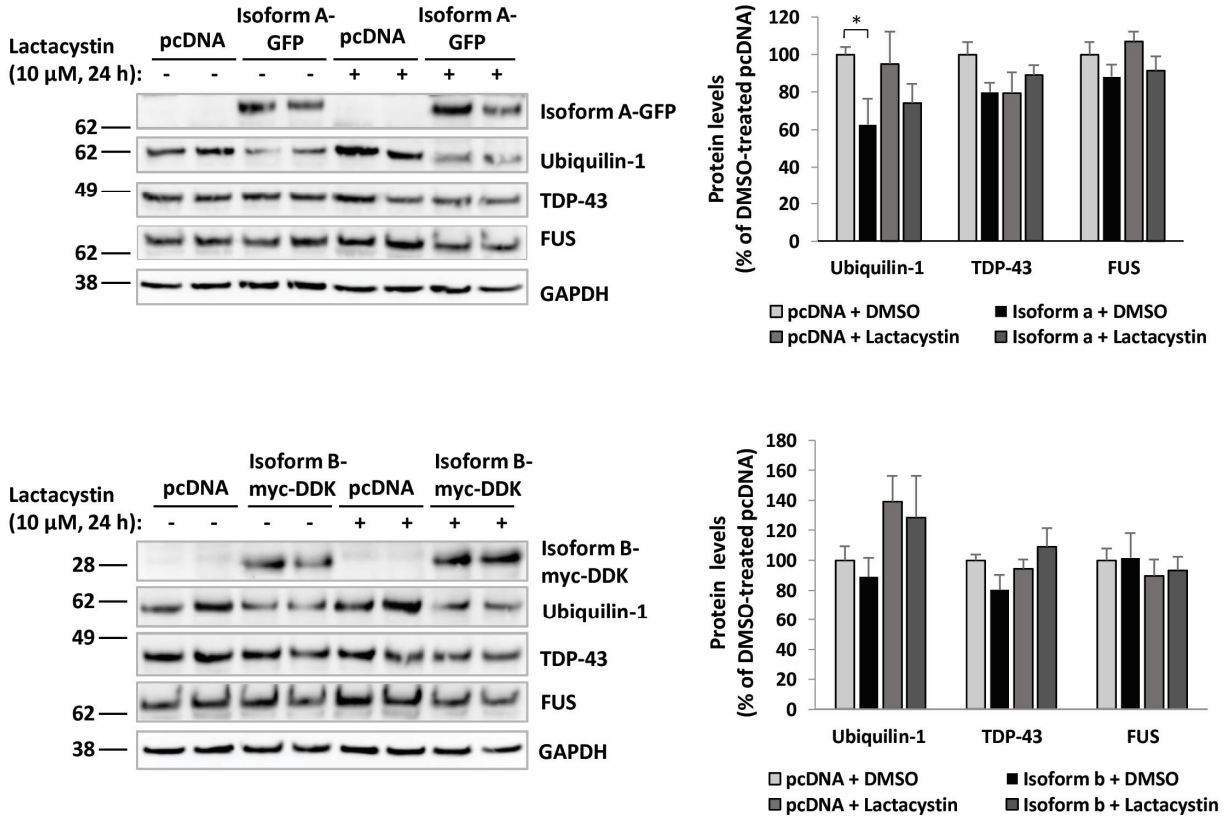

Figure 8. The levels of endogenously expressed ubiquilin-1 are significantly decreased in SH-SY5Y cells overexpressing C9orf72 isoform A. The cells were transfected with C9orf72 isoform A-GFP or B-myc-DDK and treated with proteasomal inhibitor Lactacystin (10 µM) or DMSO (vehicle) for 24 hours. Western blot showing the levels of endogenous ubiquilin-1, TDP-43, and FUS in cells overexpressing C9orf72 isoform A. Quantification of the protein levels is shown on the right. The levels of ubiquilin-1 are significantly decreased in cells overexpressing isoform A as compared to control cells (pcDNA). The decrease is also evident, but not significant, in lactacystin-treated isoform A overexpressing cells. Western blot showing the levels of endogenous ubiquilin-1, TDP-43, and FUS in cells overexpressing C9orf72 isoform B. Quantification of the protein levels is shown on the right. Molecular weights are shown on the left of the Western blots as kDa. Protein levels are normalized to those of GAPDH and show as % of the levels in DMSO-treated pcDNA samples (=100%). Mean ± SEM, n = 4. Mann Whitney U, *p < 0.05.

5.3 Levels of endogenous C9orf72 isoform **A** and **B** and other FTD-related proteins in the frontal cortex of human FTD/ALS and AD brain

The *C9ORF72* mutation status of three FTD, one ALS, and six AD human frontal cortex samples obtained from Kuopio Brain Bank was previously detected with RP-PCR at the Institute of Clinical Medicine, Neurology at the University of Eastern Finland. Three of the four FTD and ALS cases were detected to be *C9ORF72*-positive (repeat expansion carriers) and one *C9ORF72*-negative, whereas all AD cases were *C9ORF72*-negative. The protein levels of C9orf72, ubiquilin-1, TDP-43, and FUS were analyzed with Western blot and normalized to the levels of GAPDH (**Figure 9**). There were no significant differences in C9orf72, ubiquilin-1, TDP-43, or FUS levels between ORF+ and ORF- cases. Thus, it appears that there is no correlation between presence of the *C9ORF72* repeat expansion and alterations in the levels of C9orf72 or other studied FTD-related proteins in human frontal cortex. A trend of lower C9orf72 isoform B protein levels was observed in *C9ORF72* mutation carriers compared to those without it, but it did not reach statistical significance.

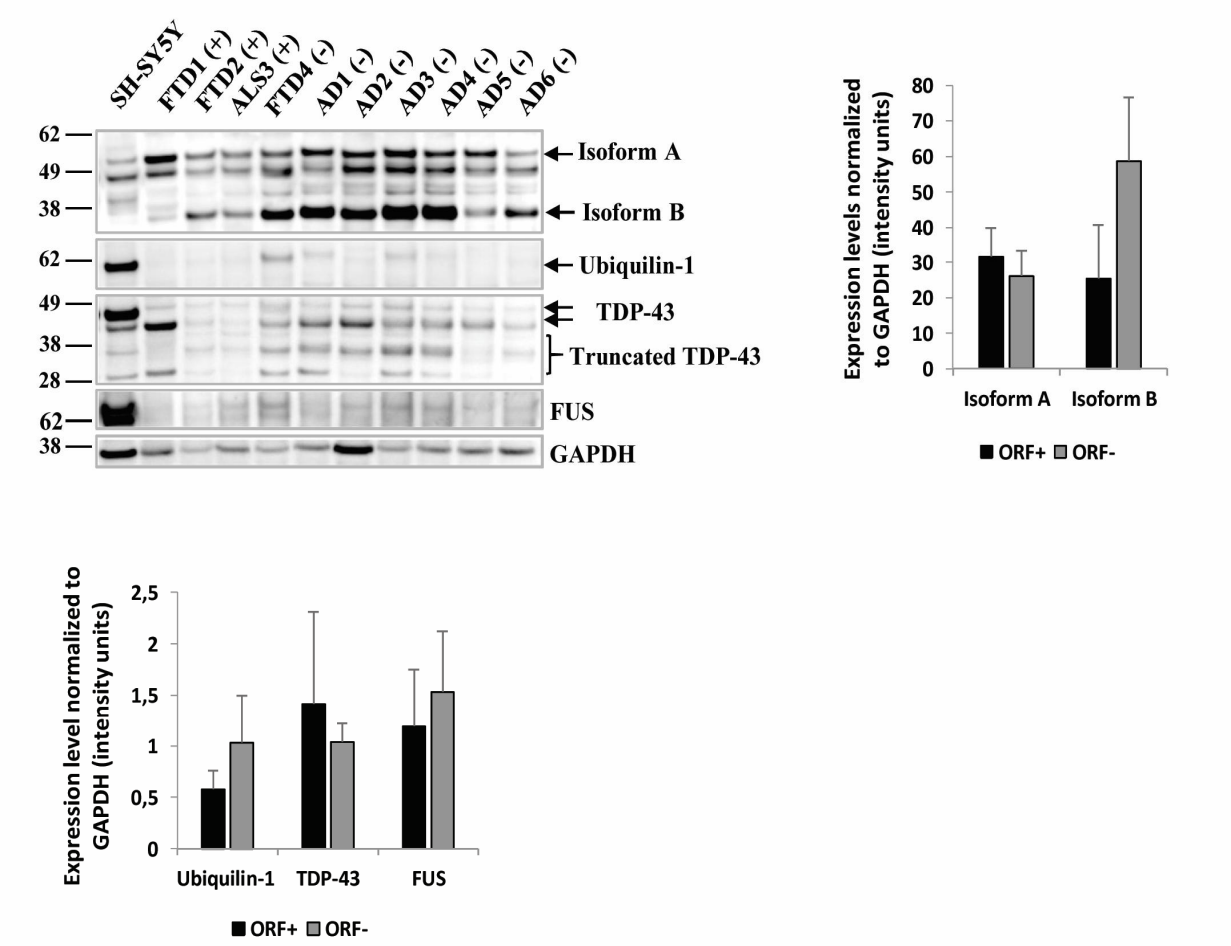

Figure 9. Expression of endogenously expressed C9orf72 isoform A and B and other FTD-associated proteins in the frontal cortex of human FTD/ALS and AD brain. Western blot showing the protein levels of C9orf72 isoform A and B, and ubiquilin-1, TDP-43, and FUS in the frontal cortex of FTD/ALS or AD brain. The cases carrying the *C9ORF72* hexanucleotide repeat expansion are indicated as (+) and negative cases as (-). SH-SY5Y cell lysate is shown in the first lane as a positive control for the proteins. Molecular weights are shown on the left of the Western blots as kDa. Quantification of the levels of ubiquilin-1, TDP-43, and FUS are shown as relative intensity units of the protein bands normalized to GAPDH intensity and expressed as mean ± SEM. ORF+, n = 3, ORF-, n = 7. Mann-Whitney U test, not significant.

6 DISCUSSION

Our understanding of the pathophysiological functions of *C9ORF72* hexanucleotide repeat expansion has grown steadily after its discovery a couple of years ago. However, the normal physiological functions of C9orf72 isoforms are not completely understood. Our results provide novel information on the expression and function of C9orf72 proteins in neuronal cells and human brain tissue.

We observed that lactacystin-induced proteasomal inhibition increases both isoform A and isoform B protein levels in neuronal SH-SY5Y-cells. This implies that the levels of these proteins are at least to some extent regulated through UPS-mediated degradation. Whether C9orf72 isoforms are degraded also through autophagy remains unclear. There were no alterations in FUS or TDP-43 protein levels in response to proteasomal inhibition, which suggest that these proteins might not be degraded through UPS. However, another study suggests that proteasomal inhibition by lactacystin leads to accumulation of TDP-43 in primary hippocampal and cortical neurons. This implies that the TDP-43 is turned over by UPS (77). Also the levels of FUS have been shown to be UPS dependent in other studies, arguing against our results (78). However, these discrepancies may be due to the use of different cells or e.g. lactacystin treatment times between the studies. Decreased ubiquilin-1 protein levels were observed in cells overexpressing the C9orf72 protein isoform A. The mechanisms underlying this finding are still unknown, but it seems, that this interaction is specific to isoform A since we did not observe a similar decrease in isoform B overexpressing cells. Further studies are needed to elucidate this potential interaction in greater detail.

Results from another research group suggest, that the levels of C9orf72 isoform A protein levels are decreased in brain tissue of expansion carriers (25). In our study, we did not observe statistically significant alterations in any of the studied protein levels, including C9orf72, in human brain tissue samples. However, we detected a trend towards decreased protein isoform B levels in expansion carriers using a commercially available antibody, but this decrease was not statistically significant. Larger number of samples would be needed to determine whether this observation is true. In addition, the specificity of the antibody which we have used is under debate and thus other antibodies should be used in further validation of the results.

It is believed that complex interplay between many genetic and environmental factors play a role in the pathology of C9FTD/ALS. It remains unknown how the repeat expansion in *C9ORF72* causes the different observed clinical phenotypes and which molecular mechanisms contribute to these differences. It would be intriguing to know whether the repeat expansion of various lengths causes different disease phenotypes through different pathological mechanisms. Development of human tissue-based cell models would be important to study the underlying pathological mechanisms in greater detail and to test potential therapeutics to treat this detrimental disease in the future. Perhaps patient-derived iPSCs could be an answer to this dilemma, since they would allow researchers to study disease-related endogenous mutations. Studies with animal models have also given promising results on using ASOs as a therapy to treat repeat expansion carriers (57). However this method

should be approached cautiously, since it must be remembered that C9FTD/ALS pathology is believed to have arisen from both loss and gain of function. In addition, animal models should be studied extensively to increase the predictive value and to facilitate the translation from experimental models to clinical trials. Thus, further studies with animal models and patient iPSC-derived cells are needed in order to unravel the normal and pathological functions of the *C9ORF72* gene and proteins. This research will provide valuable information on the normal and pathophysiological functions of the *C9ORF72* gene and proteins, and may eventually aid in the development of biomarkers and treatments for this devastating disease.

Taken together, this Master's thesis has provided novel data related to the regulation and protein interactions of C9orf72 protein isoforms as well as C9orf72 expression in human brain. However, further studies in cell-based and animal models and in human-derived cells and samples are needed for better understanding of the physiological and pathophysiological functions of *C9ORF72*.

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