THE IMPACT OF MUTANT ALSIN PROTEIN ON MOTOR NEURON MODELS AND THE EFFECTS OF ALSIN GENE KNOCK-DOWN ON MOTOR PROTEIN GENES

by

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To my amazing friend Avi

and

all other ALS patients

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ABSTRACT

THE IMPACT OF MUTANT ALSIN PROTEIN ON MOTOR NEURON MODELS AND THE EFFECTS OF ALSIN GENE KNOCK-DOWN ON MOTOR PROTEIN GENES

Alsin is a ubiquitously expressed protein with the highest levels in the central nervous system. Mutations in the alsin gene have been associated with several juvenile onset recessive motor neuron diseases. Nine out of 12 reported mutations in the alsin gene are deleterious mutations, leading to premature termination of the protein by an early stop codon; all affected patients are homozygous and all unaffected siblings are heterozygous or normal for the alsin mutations. These together, suggest a loss of function mechanism, responsible for motor neuron degeneration. The aim of this study is to understand the function of alsin using two different approaches. The first approach investigates the effects of mutant alsin on cells with motor neuron characteristics. The alsin mutation generated in this study leads to the truncation of one third of the protein, including the VPS domain, as it is the case in the majority of the natural mutations. The second approach analyzes alsin's possible interactions with six motor proteins, which have a role in axonal transport: DCTN1, DCTN2, DCTN3, KIF3A, KIF3B and KIF5A. The results of this thesis suggest a rapid degradation of the mutant alsin protein in the NSC34 cells. Thus, it concludes that the pathogenesis of ALS might be due to the overall loss of the alsin protein rather than the loss of the VPS domain, as reported before in the literature. This study also implicates a possible interaction of alsin with three of the six investigated proteins: DCTN1, KIF3A and KIF3B. It further shows a co-localization of alsin and KIF3A in the perinuclear region of N2a cells. This thesis is a first attempt in our laboratory for elucidating the alsin-interacting partners in cell culture systems; we hope that this study will help to pave the ways for understanding the complicated and multilayered functions of the alsin protein.

ÖZET

MUTANT ALSİN PROTEİNİNİN MOTOR NÖRON MODELLERİ VE SUSTURULMUŞ ALSİN GENİNİN MOTOR PROTEİN GENLERİ ÜZERİNDEKİ ETKİLERİ

Alsin, en fazla merkezi sinir sisteminde olmak üzere, tüm hücre tiplerinde sentezlenen bir proteindir. Bu gendeki mutasyonlar, erken başlangıçlı resesif bir dizi motor nöron hastalığıyla ilişkilendirilmiştir. Alsin geninde tanımlanmış 12 mutasyondan dokuzu delesyon mutasyonlarıdır, erken stop kodon oluşumuna neden olarak, protein translasyonunu zamanından evvel sonlandırırlar. Alsin mutasyonu taşıyan tüm hastaların homozigot, etkilenmemiş aile bireylerinin heterozigot veya normal olmaları, motor nöronlardaki dejenerasyonunun alsinin işlev kaybından kaynaklandığını işaret etmektedir. Bu çalışma, iki farklı yaklaşımla, alsinin işlevlerini anlamayı amaçlamaktadır. İlk aşamada, mutant alsinin motor nöron özelliği taşıyan hücrelerdeki etkileri araştırılmıştır. Çalışmada kullanılan alsin mutasyonu, doğal mutasyonların çoğunluğu gibi, alsinin VPS bölgesinin eksik olduğu bir protein oluşumuna neden olmaktadır. İkinci aşamada, alsinin bir dizi motor proteini ile olası etkileşimleri araştırılmıştır. Aksonal transportta rol oynayan proteinlerin (DCTN1, DCTN2, DCTN3, KIF3A, KIF3B ve KIF5A) seçilme nedeni, alsinin tüm hücrelerde sentezlenmesine rağmen, dejenerasyonun sadece motor nöronlara sınırlı kalmasından dolayıdır. Bu tezin sonuçları, mutant alsinin NSC34 hücrelerinde hızlı bir şekilde yıkıldığı ve hastalık oluşumunun, literatürde belirtildiği gibi, yalnız VPS bölgesinin değil, tüm alsin proteininin yokluğundan kaynaklı olabileceği yönündedir. Çalışma, alsin susturulduğunda, DCTN1, KIF3A ve KIF3B genlerinin anlatım düzeylerindeki farklılıklara dayanarak, bu proteinlerin alsin ile olası bir etkileşimlerinin olduğuna işaret etmekte, Ayrıca alsinin, kinezinlerin protein etkileşim bölgesi olan KIF3A ile kolokalize olduğunu da göstermektedir. Alsin ve etkileştiği proteinlerin, laboratuvarımızda hücre kültürü sisteminde ilk defa araştırıldığı bu çalışma ve devamının, alsinin hücredeki karmaşık ve çok-yönlü işlevlerine ışık tutması beklenmektedir.

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LIST OF ABBREVIATIONS

O_2^-	Superoxide
^oC	Centigrade degree
V	Volt
v	Volume
w	Weight
µg	Microgram
µl	Microliter
AD	Autosomal Dominant
ALS	Amyotrophic Lateral Sclerosis
ALS2	Alsin
ALS-FTD	Amyotrophic Lateral Sclerosis-Frontotemporal Dementia
ANG	Angiogenin
APS	Amonium Persulfate
AR	Autosomal Recessive
AT	Axona Transport
BSA	Bovine Serum Albumine
CBB	Coommassie Brilliant Blue
CNS	Central Nervous System
cnt	Control
CO_2	Carbondioxide
Cu	Cupper
Cu^{+2}	Cupper Ion
Dbl	Diffuse B Cell Lymphoma
DCTN	Dynactin
DCTN1	Dynactin 1
DCTN2	Dynactin 2
DCTN3	Dynactin 3

DH	Diffuse B Cell Lymphoma Homologous Domain
DMEM	Dulbecco’s Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
EAAT2	Excitatory Amino Acid Neurotransmitter 2
EDTA	Ethylenediaminetetraacetic Acid
EEA1	Early Endosome Antigen 1
fALS	Familial Amyotrophic Lateral Sclerosis
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
fs	Frameshift
FTD	Frontotemporal Dementia
FTLD	Frontotemporal Lobar Degeneration
FUS	Fused in Sarcoma
GDP	Guanosine Diphosphate
GEF	Guanine-nucleotide Exchange Factor
GTP	Guanosine Triphosphate
H_2O	Water
H_2O_2	Hydrogen Peroxide
HS	Horse Serum
HSP	Hereditary Spastic Paraplegia
IAHSP	Infantile-onset Ascending Hereditary Spastic Paraplegia
IF	Immunoflourescence
IgG	Immunoglobulin G
IP	Immunoprecipitation
kb	Kilobase
kDa	Kilodalton
KIF3A	Kinesin-family Member 3A
KIF3B	Kinesin-family Member 3B
KIF5A	Kinesin Heavy Chain Isoform 5A
KIFs	Superfamily of Kinesin proteins
LB	Lysogeny Broth
LMN	Lower Motor Neuron
LMND	Lower Motor Neuron Disease

mA	Miliamper
MEM	Modified Eagle Medium
mg	Miligram
$MgCl_2$	Magnesium Chloride
min.	Minutes
ml	Mililiter
mm	Millimeter
MN	Motor Neuron
MORN	Membrane Occupation and Recognition Nexus
ms	Missense
mt	Mutant
N2a	Neuroblastoma 2A
NaCl	Sodium Chloride
NEAA	Non-esential Amino acid
NF	Neurofilaments
NF-H	Heavy Chain Neurofilament
NF-M	Medium Chain Neurofilament
ng	Nanogram
NO_3^-	Peroxynitrite
ns	Nonsense
NSC34	Neuroblastoma-Spinal Cord Hybrid Cell Line
OH^-	Hydroxyl radicals
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
Pen/Strep	Penicillin/Streptomycin
PH	Pleckstrin Homology Domain
PLS	Primary Lateral Sclerosis
PLSJ	Juvenile Primary Lateral Sclerosis
pmole	Picomole
qRT-PCR	Real-time Polymerase Chain Reaction
Rab5	Ras-related in Brain
Rac1	Ras-related C3 Botulinum toxin Substrate 1
Ran	Ras-related Nuclear GTPase

Ras	RAt Sarcoma Subfamily
RCC1	Regulator of Chromosome Condensation 1
Rho	Ras Homologous Member
RIPA	Radio Immunoprecipitation Assay
RLD	RCC1-like Domain
rpm	Rotations per Minute
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
sALS	Sporadic Amyotrophic Lateral Sclerosis
SDS	Sodium Dedocyl Sulfate
SDS-PAGE	SDS- Polyacrylamide Gel Electrophoresis
sec.	Seconds
SETX	Senataxin
shRNA	Short Hairpin RNA
SOD1	Superoxide Dismutase1
TBS	Tris Buffered Saline
TBST	Tris Buffered Saline Tween
TDP-43	TAR DNA binding protein
TEMED	Tetramethylethylenediamine
TWEEN	Polysorbate
VABP	Vesicle-associated Membrane Protein-associated Protein B
VEGF	Vascular Endothelial Growth Factor
VPS9	Vacuolar Protein Sorting 9
WB	Western Blot
wt	Wild-Type
x g	Times Gravity
Zn	Zinc
Zn^{+2}	Zinc Ion

1. INTRODUCTION

Amyotrophic Lateral Sclerosis (ALS) is a late-onset, rapidly progressive, neurodegenerative disorder. It was first described in 1869 by the French neurologist Jean-Martin Charcot (Charcot *et al.*, 1869). Today, it is also known as Motor Neuron Disease or Lou Gehrig's disease, after the death of the famous American baseball player from ALS in 1941 (Cleveland and Rothstein, 2001).

ALS is generally associated with selective degeneration of both upper and lower motor neurons (MNs) in the brain, brainstem and spinal cord (Figure 1.1.). Dysfunction of upper MNs leads to spasticity and hyperflexia, whereas lower MN degeneration triggers muscle atrophy and paralysis (Gonzalez de Aguilar *et al.*, 2007). Diagnosis is defined by clinical features described in the El Escorial (Brooks *et al.*, 2000) and Awaji Criteria (de Carvalho *et al.*, 2008).

ALS has a prevalence (total number of cases at a given time) of 4-6 per 100,000, and the incidence (number of new cases in a year) is 1-2 per 100,000. The incidence is fairly uniform throughout the world with some exceptional foci like the island of Guam and the Kii peninsula, where it is higher (Cox *et al.*, 2002). Although ALS is mostly described as late-onset, juvenile forms are also observed. Final stage of the disease is the denervation of respiratory muscles, which mostly occurs within five years of developing the disease and leads to death.

Approximately 10 per cent of all ALS cases are inherited and referred to as familial ALS (fALS); the remaining 90 per cent are sporadic ALS (sALS) without any family history. These two forms of the disease produce similar pathological hallmarks, including muscle weakness, atrophy, and spasticity. However, some minor differences are present; age of onset is almost a decade earlier and sensory neurons are more vulnerable in fALS cases, whereas the male: female ratio is slightly higher and the symptoms start more frequently in the lower extremities in sALS (Cleveland and Rothstein, 2001; Pasinelli and Brown, 2006).

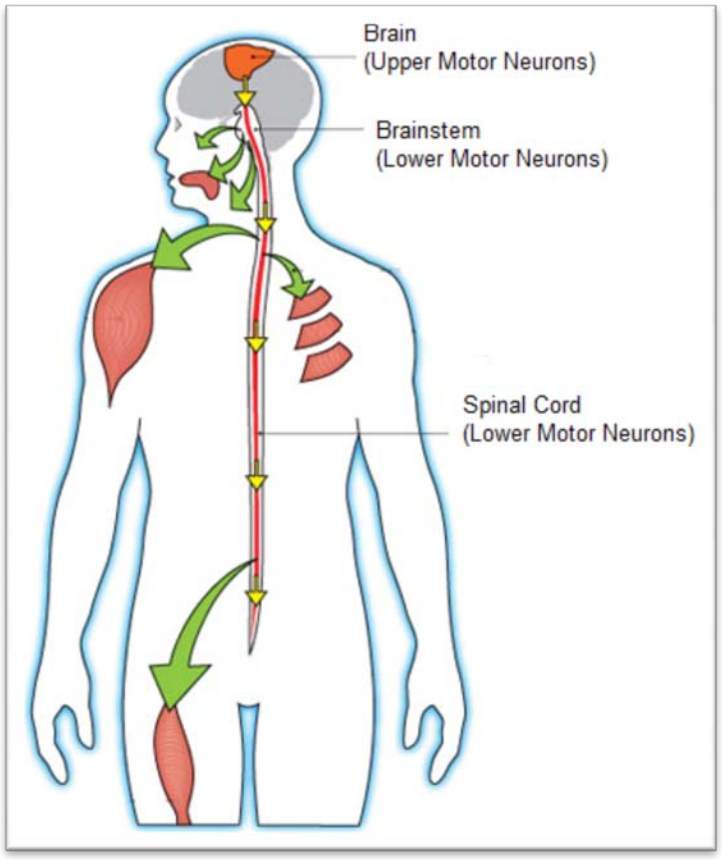

Figure 1.1. Affected regions in ALS (www.als-mda.org).

Discovery of mutations detected in various genes that have been related to ALS, provided opportunities to develop model systems for investigating the disease mechanism. Even though there are several suggested mechanisms arising from these models, the exact causes of many ALS cases are as yet undefined. The most probable explanation for this may be that, ALS is a heterogeneous disorder with multiple factors underlying the disease mechanism.

1.1. Molecular Genetics of ALS

An important breakthrough was achieved with the discovery of missense mutations in the gene encoding the Cu/Zn Superoxide Dismutase 1 (SOD1) (Rosen *et al.*, 1993). SOD1, which is the primary cause of 20 per cent of fALS cases, has been related to a dominant pattern of inheritance, with an exception of D90A mutation, which is represented in a recessive manner in the Scandinavian population (Andersen *et al.*, 1995).

SOD1 is a small protein, consisting of 153 amino-acids. It is ubiquitously expressed and highly abundant in neurons. The function of this cytosolic homodimer is to convert superoxide (O_2^-), produced by errors of oxidative phosphorylation in mitochondria, to hydrogen peroxide (H_2O_2) and then to water (H_2O) by either glutathione peroxidase or catalase (Figure 1.2.) (Cleveland, 1999).

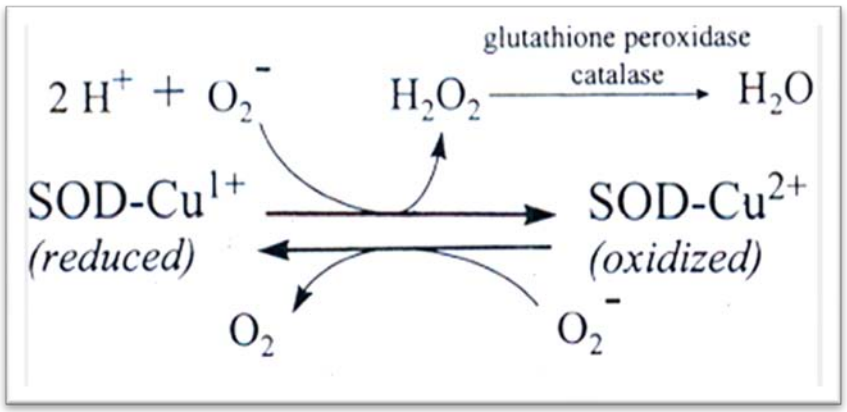

Figure 1.2. Conversion of the superoxide anion by SOD1 (Cleveland, 1999).

Following SOD1, other mutations associated with ALS were also discovered in several genes including, alsin (ALS2) (Hadano *et al.*, 2001; Yang *et al.*, 2001), VAMP-associated protein B (VAPB) (Nishimura *et al.*, 2004), senataxin (SETX) (Chen *et al.*, 2006), angiogenin (ANG) (Greenway *et al.*, 2006), dynactin1 (DCTN1) (Puls *et al.*, 2003), and two recent genes coding for DNA/RNA binding proteins, TAR DNA-binding protein (TDP-43;~3% familial cases and ~1.5% sporadic cases) (Van Deerlin *et al.*, 2008) and,. fused in sarcoma (FUS; ~4% of familial cases) (Kwiatkowski *et al.*, 2009; Vance *et al.*, 2009).

Despite of the increasing number of genes identified in ALS (Table 1.1.), majority of research is still based on SOD1-related mechanisms, since the animal models of SOD1 are the best disease models achieved up to date. There are 148 mutations identified in the SOD1 gene in humans (Jonsson *et al.*, 2009). Three of these mutations ($SOD1^{G85R}$, $SOD1^{G37R}$ and $SOD1^{G93A}$) have been widely characterized in transgenic models of ALS. Several transgenic mice studies revealed that, mutant SOD1 mice develop progressive motor neuron disease, despite the markedly elevated or unchanged SOD1 activity levels (Gurney *et al.*, 1994; Wong *et al.*, 1995; Ripps *et al.*, 1995; Bruijn *et al.*, 1997; Reaume *et*

al., 1996). These evidences indicate the contribution of mutant SOD1 in ALS in a manner of toxic gain-of-function, regardless of the amount of mutant SOD1 activity.

Table 1.1. Genes responsible for ALS (adapted from Swarup and Julien, 2010).

fALS type*	Locus	Gene	Inheritence	Clinical Pattern	Mutations	Causes sALS
ALS1	21q	SOD1	AD	Classical	>148	Yes
ALS2	2q33	ALSIN	AR	Juvenile	12	No
ALS3	18q21	?	AD	Classical	?	?
ALS4	9q34	SETX	AD	Juvenile	3	?
ALS5	15q15	?	AR	Juvenile	?	?
ALS6	16q21	FUS/TLS	AD	Classical	14	?
ALS7	20ptel-p13	?	AD	Classical	?	?
ALS8	20q13.3	VAPB	AD	Varied	1	No
ALS-FTD	9q21-q22	?	AD	With FTD	?	?
ALS-FTD	9q21.3	?	AD	With FTD	?	?
ALS	14q11.2	ANG	AD	Classical	6	Yes
ALS	1	TDP-43	AD	ALS	30	Yes
LMND	2p13	DCTN1	AD	LMND	1(+4 in ALS)	?

Genes involved in RNA metabolism

Genes involved in trafficking and transport

*ALS: amytrofic lateral sclerosis, FTD: fronto-temporal dementia, LMND: lower motor neuron disease

*AD: autosomal dominant, AR: autosomal recessive

As in the case of SOD1, in almost all examples of inherited neurodegenerative diseases, the mutant gene product is ubiquitously expressed. Several studies have converged to demonstrate that the toxicity in affected MNs in ALS is non-cell-autonomous, and developing the disease requires expression of mutant SOD1 not only in neurons, but also in non-neuronal cells (Pramatarova *et al.*, 2001; Lino *et al.*, 2002; Gong *et al.*, 2000). Clement *et al.* (2003) demonstrated that chimeric mice, that ubiquitously express mutant SOD1, can escape degeneration, if MNs are surrounded with wild-type non-neuronal cells. In another set of chimeric mice, mutant SOD1-expressing non-neuronal cells can transfer the toxic damage to wild-type MNs. These data show that cellular neighborhood is very important, and there is a definite contribution of the surrounding cell types to motor neuron degeneration.

1.2. Proposed Mechanisms in ALS

There are several mechanisms proposed for the mutant SOD1-mediated ALS (Figure 1.3.). The first one is the aberrant chemistry and the oxidative stress. It proposes that the altered SOD1 enzyme activity leads to a clumsy handling of Cu/Zn ions, which eventually results in abnormal substrate binding. Inappropriate substrates can produce peroxynitrite (NO_3^-) and/or hydroxyl radicals (OH^-). Peroxynitrite can nitrate tyrosin residues and damage proteins in affected cells, on the other hand OH^- is highly reactive (Beckman *et al.*, 1994).

Protein aggregation is a common feature of SOD1-mediated toxicity as it is in many other neurodegenerative diseases. It is reported that in ALS patients and mouse models, cytoplasmic inclusions, which are highly immunoreactive for SOD1, are present (Bruijn *et al.*, 1998). Protein aggregates can damage MNs through several mechanisms; aberrant chemistry, loss of protein function, depletion of the chaperons, dysfunction of the proteosome and inhibition of specific organelles like mitochondria and peroxisomes (Bruijn *et al.*, 2004).

In spinal MNs of $SOD1^{G93A}$ and $SOD1^{G37R}$ mice, there appeared to be vacuolated mitochondria remnants, which pointed mitochondria as a possible target in ALS (Dal Canto and Gurney, 1994; Kong and Xu, 1998; Wong *et al.*, 1995). Later, an almost 25 per cent decrease was reported in mitochondrial enzymatic activity in spinal cord extracts obtained from sALS patients. This decrease could be caused by the mutant SOD1 which is reported to be present in spinal cord mitochondria's intermembrane space (Mattiazzi *et al.*, 2002).

Despite the unknown primary cause for degenerating MNs, the final event in the death has been partly clarified. Months before neuronal death and phenotypic expression of the disease symptoms, caspase 1 is activated in SOD1 mutants. After caspase 1, caspase 3 (a cysteine aspartate protease, responsible for degradation of many ingredients in apoptotic cell death) is activated as a central feature in MNs and astrocytes before cell death (Pasinelli *et al.*, 2000; Vukosavic *et al.*, 2000).

Glutamate is the major excitatory neurotransmitter in the central nervous system (Brown *et al.*, 2001); glial glutamate transporter, EAAT2, clears the bulk of glutamate in the synapses. Glutamate-mediated excitotoxicity due to the repetitive firing of calcium-permeable glutamate receptors, by the excess amount of calcium, released to the synapse, is implicated in MN death (Rothstein *et al.*, 1996). Loss of the astroglial EAAT2 protein, results in increased glutamate levels, and has been detected in 40 per cent of ALS patients (Rothstein *et al.*, 1995). The spinal MNs at risk are shown to be reduced in calcium binding components, resulting in a higher amount of calcium and glutamate, leading to excitotoxicity (Alexianu *et al.*, 1994; Ince *et al.*, 1993). This hypothesis is supported by the fact that the only FDA (Food and Drug Administration)-approved therapy for ALS, Riluzole, functions by decreasing the glutamate toxicity.

Another component proposed in the selective vulnerability, is the abnormal assembly of neurofilaments (NFs) in the axons, which is very essential for the establishment of the proper MN structure. NF tangles are a common pathological hallmark in many neurodegenerative diseases, including sALS (Hirano *et al.*, 1984) and fALS (Hirano *et al.*, 1991) Reduction of axonal NFs or the increase of the NF-H and NF-M (NF heavy and medium chains: together responsible for forming proper NF assemblies), slows down the onset of mutant SOD1-mediated disease (Couillard-Despres *et al.*, 1998). Deletions and insertions in the repetitive tail domain of the NF-H has been identified in 1 per cent of 1300 examined ALS patients (Al-Chalabi *et al.*, 1999; Tomkins *et al.*, 1998; Figlewicz *et al.*, 1994).

Due to the asymmetric geometry and the large volume of MNs, axonal transport is very crucial for transferring the synthesized components and organelles from the cell body to the extended axonal end. Reduced transport correlates with motor neuron disease. Williamson *et al.* showed that mutant SOD1 impairs slow axonal transport months before the disease onset (Williamson *et al.*, 1998). (Axonal transport will be discussed in detail in Section 1.4.).

Microglia involvement has been described in many neurological disorders. Although there is little known about the microglial involvement in ALS, microglial activation has been shown in ALS tissue with MN loss (Kwamata *et al.*, 1992; Ince *et al.*, 1996). Also,

pharmacological studies on Minocyclin, an antibiotic which blocks microglia activation, displayed delay in disease progression in mice models (Yrjanheikki *et al.*, 1999).

Finally, a completely unexpected contributor to ALS emerged from the ALS-like disease developing mice with a targeted deletion in the vascular endothelial cell growth factor (VEGF) gene (Oosthuyse *et al.*, 2001). VEGF controls the growth and permeability of blood vessels, its exact mechanism in ALS pathology is still unsolved. Further validation is strongly needed to show that VEGF, but not any other gene with close association, is the key gene contributing to ALS (Bruijn *et al.*, 2004).

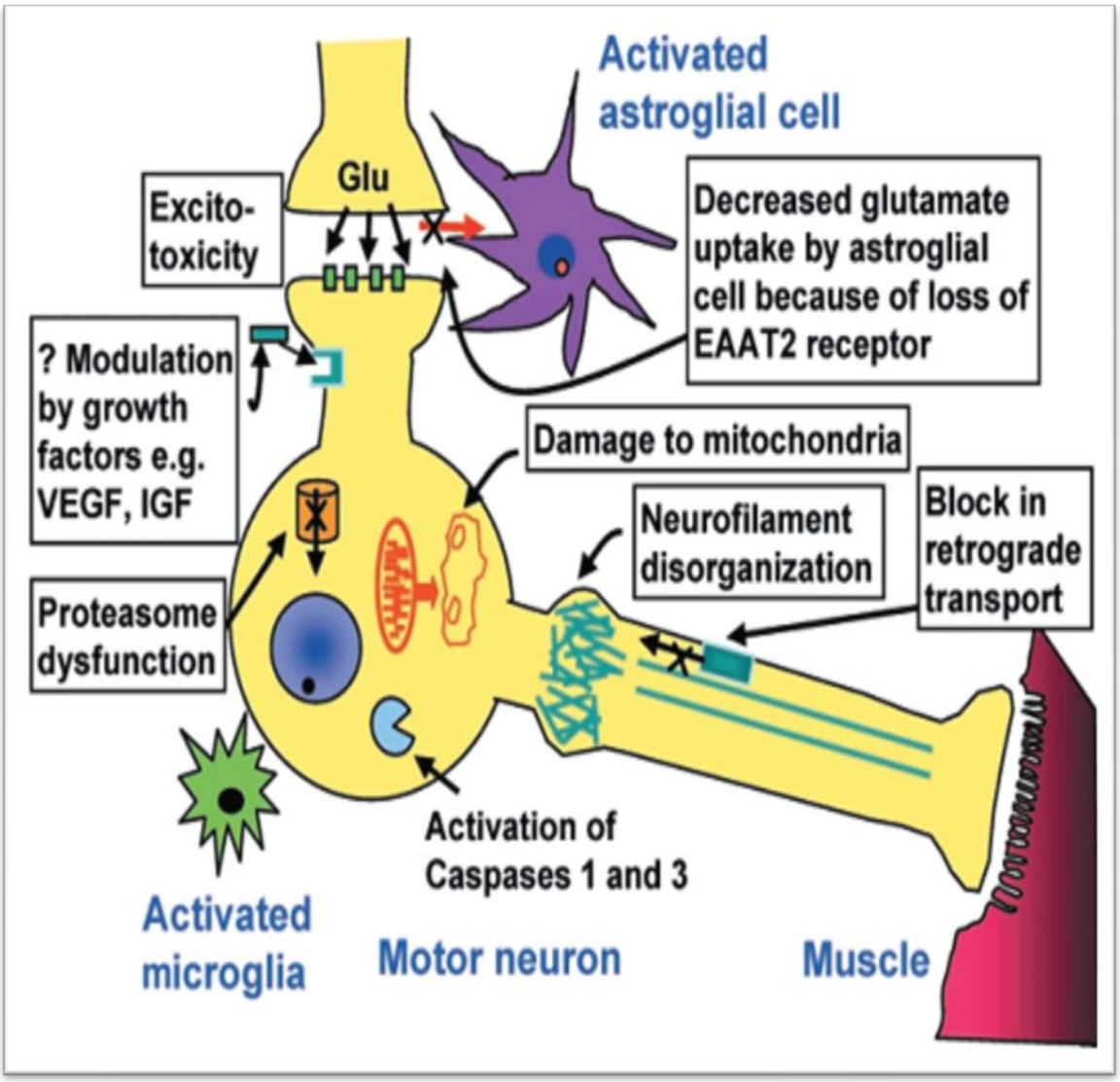

Figure 1.3. Proposed ALS mechanisms (Bruijn *et al.*, 2004).

1.3. Alsin

Alsin (ALS2) is a ubiquitously expressed protein with the highest levels in the central nervous system (CNS), mainly in cerebellum. The alsin gene located on chromosome 2q33, contains 33 introns and 34 exons, coding for a 184 kDa protein consisting of 1657 amino acids. alsin has also a shorter transcript due to its alternative splicing, a 396 amino acid protein. (Hadano *et al.*, 2001).

1.3.1. Alsin Mutations and Motor Neuron Diseases

Mutations in the alsin gene have been associated with some juvenile onset recessive motor neuron diseases, including a type of juvenile Amyotrophic Lateral Sclerosis (ALS2), Juvenile Primary Lateral Sclerosis (PLSJ) (Hadano *et al.*, 2001; Yang *et al.*, 2001) and Infantile-onset Ascending Hereditary Spastic Paraplegia (IAHSP) (Devon *et al.*, 2003). Nine out of 12 reported mutations in the alsin gene are deleterious mutations leading to the premature termination of the protein by an early stop codon (Table 1.2.). Also, all affected patients are homozygous and all unaffected siblings are heterozygous or normal for the alsin mutations (Gros-Louis *et al.*, 2003). These together, suggest a loss of function mechanism, responsible from the MN degeneration.

Primary Lateral Sclerosis (PLS) and Hereditary Spastic Paraplegia (HSP) are disorders in which MN pathway is affected differently than in ALS (Table 1.3.). ALS is a progressive disease that involves upper and lower MNs, while in PLS only upper MNs are affected and the clinical course is sufficiently different from ALS. HSP also has its distinctive clinical phenotypes described by Harding in 1981. All three diseases have been categorized into two, as pure and complicated forms based on absence or presence of additional neurological or non-neurological clinical features (in case of ALS, they are referred as the SOD1-mediated form and ALS-like diseases). The clinical features used to separate these three disorders are depicted in Table 1.3.

PLS is a slowly progressive, sporadic disorder of adulthood (average age of onset is 53.4 years). However, juvenile cases have been described in both isolated and familial forms (Gascon *et al.*, 1995; Lerman-Sagie *et al.*, 1996). PLS has also been defined, as a

point in spectrum of multisystem neurodegenerative disorders, since it is proposed to convert to ALS after up to 27 years in some cases (Verma *et al.*, 2001). Thus far, two frameshift and one missense mutations in alsin have been related to PLS (Table 1.2.).

Table 1.2 Mutations in Alsin and corresponding disorders
(adapted from Hadano *et al.*,2007).

Mutations (Transcript or gene)[a]	**Location**	**Type**[b]	**Origin**	**Disease**[c]	**Phenotype**[d]
c.138delA	Exon 3	fs	Tunisian	ALS2	U/L
c.470G>A	Exon 4	ms	Turkish	IAHSP	U
c.553delA	Exon 4	fs	Turkish	ALS2	U/L
c.1007_1008delTA	Exon 4	fs	Italian	IAHSP	U
c.1425_1426delAG	Exon 5	fs	Kuwaiti	PLSJ	U
IVS5(c.1472)-1G> T(C.1472_1481del TTTCCCCCAG)	Intron 5	fs	French	IAHSP	U
c.1619G>A	Exon 6	ms	Italian	PLSJ	U
c.1867_1868delCT	Exon 9	fs	Saudi Arabian	PLSJ	U
c.2537_2538delAT	Exon 13	fs	Italian	IAHSP	U
c.2992C>T	Exon 18	ns	Israeli	IAHSP	U
c.3619delA	Exon 23	fs	Algerian	IAHSP	U
c.4721delT	Exon 32	fs	Pakistani	IAHSP	U

[a] The description of the mutations are accorded by den Dunnen and Antonarakis (Hum Mut 15, 7-12, 2000).

[b] fs: frameshift, ms: missense, ns: nonsense.

[c] ALS2: amytrophic lateral sclerosis 2 (OMIM 205100), PLSJ: juvenile primary lateral sclerosis (OMIM 606353), IAHSP: infantile-onset ascending hereditary spastic paralysis (OMIM 607225).

[d] U: upper motor neuron involvement, L: lower motor neuron involvement.

HSP is a heterogeneous group of disorders with a common phenotype of progressive spasticity. The onset is ranged between infantility and the eighth decade of life (Harding *et al.*, 1981). The obligatory feature of HSP is the presence of a family history. There are autosomal dominant, autosomal recessive and X-linked inheritance patterns deriving from mutations in several well-identified genes. Mutations in the spastin and atlastin genes account for approximately 50 per cent of all dominantly inherited HSP cases (Strong and Gordon, 2005). While being symptomatically uncommon, an adult onset HSP with

amyotrophy is due to an autosomal dominant mutation in KIF5A (Fink *et al.*, 2003). In addition to pure HSP-causing mutations, five frameshift, one missense and one nonsense mutations in alsin have been reported in IAHSP (Table 1.2.).

Table 1.3. Classification of clinical features of PLS, HSP and ALS (adapted from Strong and Gordon, 2005).

Clinical features		
Disease:	**Pure**	**Complicated**
PLS	> Insidiously progressive spasticity with only slight weakness; may be asymmetric in presentation > Spastic dysarthria > Pseudobulbar affect	> Cognitive dysfunction consistent with FTLD*, including presence of tau immunoreactive aggregates > Amyotrophy with active denervation/renervation (electromyographic, muscle biopsy), with or without fasciculations (th latter are not sufficient for a diagnosis of ALS) > Parkinsonism (extrapyramidial signs) > Subclinical sensory dysfunction > Neuropathological features of MN degeneration tupical of ALS (Bunina bodies; ubiquitinated inclusions) > Bladder involvement (rare and late)
HSP	> Positive family history > Progressive gait disturbance > Lower limb spasticity	> Distal amyotrophy > Prominent ataxia > Peripheral neuropathy (axonal) > Extrapyramidal dysfunction; choreoathetosis > Dementia (reduced recent memory, attention, perceptual speed, visuomotor control) > Mental retardation > Pigmentary macular degeneration; optic neuropathy > Misc. (cataracts, shoert stature, ichthyosis, deafness)
ALS	> Lower motor neuron dysfunction > Upper motor neuron dysfunction > Progressive spread of symptoms	> Cognitive dysfunction consistent with FTLD*, including presence of tau immunoreactive aggregates > Extra pyramidal signs (bradykinesia, cogwheel rigidity, tremor) > Cerebellar degeneration > Autonomic dysfunction (abnormal cardiovascular reflexes, bowel or bladder dysfunction) > Ocular movement abnormalities
* FTLD- Frontotemporal Lobar Degeneration		

1.3.2. Protein Structure and Function of Alsin

GTPases are switches, that cycle between an inactive guanosine diphosphate (GDP) and an active guanosine triphosphate (GTP)-bound state; they regulate a wide range of cellular and molecular processes (Vetter and Wittinghofer, 2001). Guanine-nucleotide exchange factors (GEF) activate GTPases by associating with the GDP-bounded form, and mediating GDP dissociation and GTP binding. The full length alsin protein contains three guanine-nucleotide exchange factor (GEF) domains, based on the sequence homology. All GEFs functionally resembling to alsin are activators of the RAt Sarcoma (Ras) subfamily, which is a group of small GTPases (Hadano *et al.*, 2001; Yang *et al.*, 2001).

The N-terminal region of alsin is highly homologous to the regulator of chromosome condensation (RCC1), a GEF for the Ras-related nuclear (Ran) GTPase. This alsin domain is referred to as RCC1-like domain (RLD). However, no significant alsin-associated Ran-GEF activity has been detected *in vitro* yet. RLDs have been identified in many other proteins with diverse functions, therefore it is likely that, alsin-RLD might function as a protein interacting domain (Hadano *et al.*, 2007).

The middle portion of alsin contains two domains: a diffuse B cell lymphoma (Dbl) homologous domain (DH) and a pleckstrin homology domain (PH) (Hadano *et al.*, 2001; Yang *et al.*, 2001). The Dbl and pleckstrin domains are hallmarks of the GEFs that activate Ras homologous member (Rho)-type GTPases (Rossman *et al.*, 2005). Rho subfamily proteins are critical regulators of the actin cytoskeleton organization, various signaling cascades and neuronal morphogenesis. Further, they have emerged as major players in vesicle budding, trafficking, fusion and signal transduction (Miaczynska *et al.*, 2004).

The C-terminal region of alsin contains a vacuolar protein sorting 9 (VPS9)-like domain, which has been found in Ras-related in brain 5 (Rab5) GEFs, that are involved in vesicle endocytosis, trafficking and early endosomal membrane fusion. (Carney *et al.*, 2006). In addition, there have been eight membrane occupation and recognition nexus (MORN) motifs, identified in between the DH/PH and VPS9 regions of the alsin protein. MORN motifs are implicated in binding of the plasma membrane (Takeshima *et al.*, 2000). Previous studies have shown that VPS9 domain of alsin together with its upstream MORN

motifs can activate the Rab5 GTPases and this function is promoted by the DH/PH domain (Otomo *et al.*, 2003; Topp *et al.*, 2004).

The function of alsin is assumed to be regulating/activating multiple small GTPases, as it contains putative GEF domains: RLD, DH/PH and VPS9 (Figure 1.4.). Additive function hypothesis arose from further studies of alsin. Immunocytochemical studies at high magnification detected alsin, distributed in the cytoplasm in a diffused manner. However, consistent presence of several dot staining in soma, dendrites and axons has been demonstrating the presence of alsin in endosomal compartments, in the neurons. On the other hand, alsin is shown to co-localize with endosome markers; Rab5 and Early Endosome Antigen 1 protein (EEA1), and it is enriched on the tip of axons with a dense localization to a Rho GTPase, Rac1, and vesicles at growth cones (Otomo *et al.*, 2003; Hadano *et al.*, 2007). Currently endosomal trafficking, axonal guidance and outgrowth has emerged as potential functions of alsin, but several other mechanisms can be considered according to the interacting proteins of the RLD domain of the alsin (Figure 1.5.).

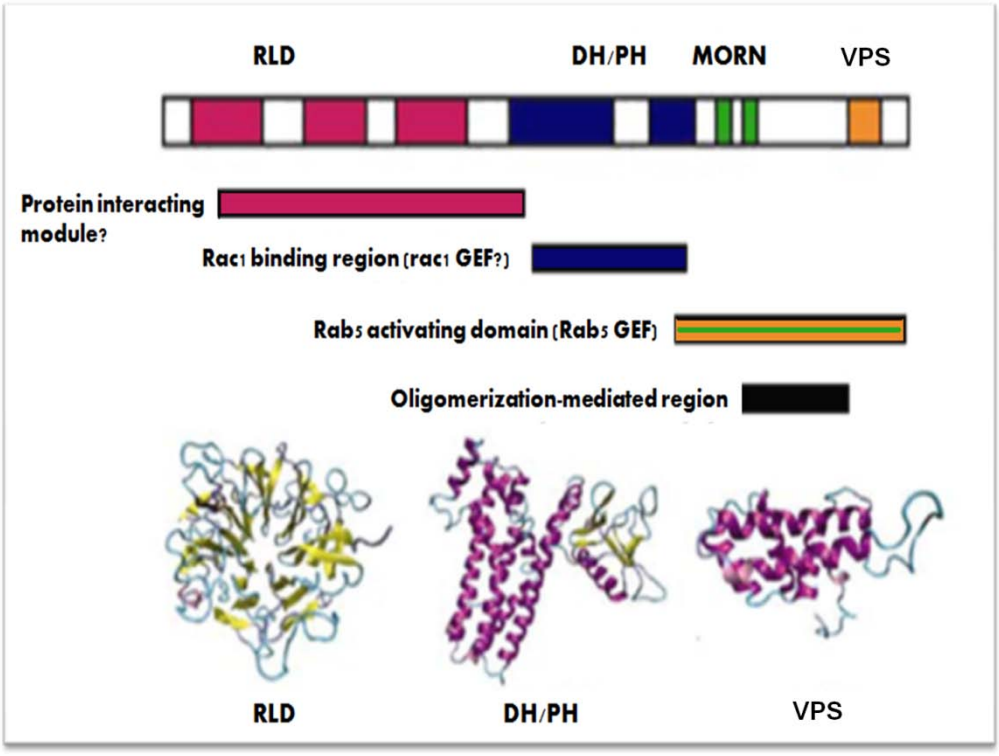

Figure 1.4. Schematic representation of full length human alsin protein and possible functions of its regions (adapted from Chandran *et al.*, 2007).

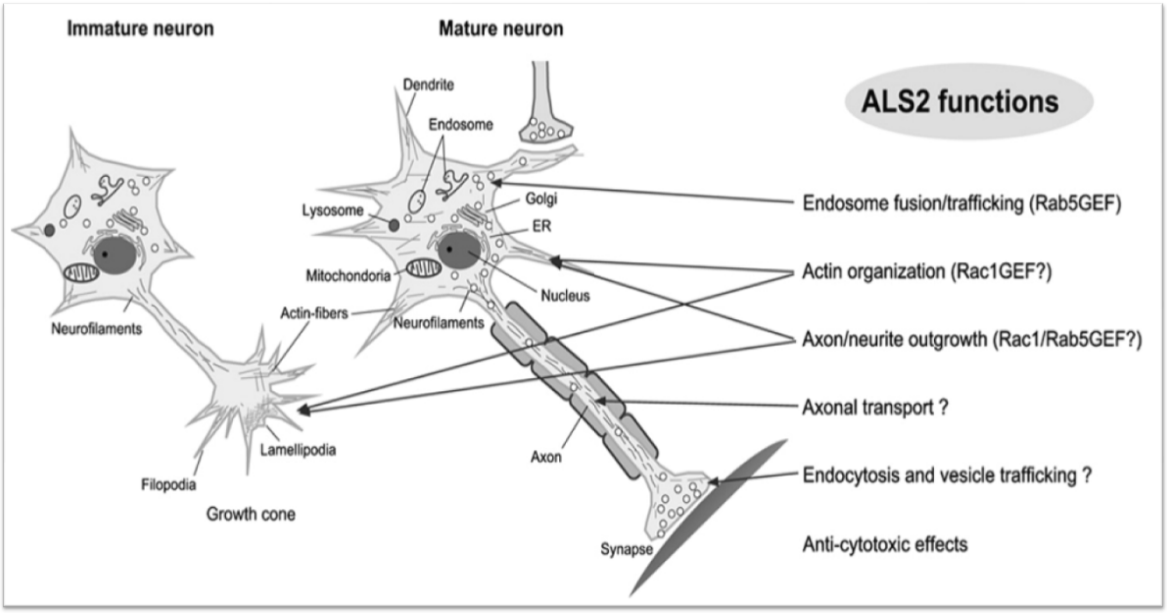

Figure 1.5. Proposed functions of alsin in developing and matured neurons (Hadano *et al.*, 2007).

While the exact etiology of motor neuron diseases is still unknown, perturbations in molecular and vesicular trafficking have emerged as a major pathogenic mechanism. SOD1 transgenic mice have been reported to develop aberrant axonal transport in the MNs. Loss of the alsin in alsin knock-out mice, damages Rab5-dependent endocytosis, whereas Alsin over-expression in cell cultures, causes severe phenotypic changes like enlargement and accumulation of early endosomes and impairment of mitochondrial trafficking. (Hadano *et al.*, 2006; Devon *et al.*, 2006; Millecamps *et al.*, 2005). In the last few years several genes, associated with intracellular or axonal transport, have been shown to have mutations leading to various types of MN dysfunction (Chandran *et al.*, 2007). For example, mutations in motor proteins such as dynein, dynactin subunit 1 (DCTN1) and the kinesin family protein KIF5A are also linked to motor neuron diseases, supporting the importance of axonal transport (Puls *et al.*, 2005; Reid *et al.*, 2002; Hafezparast *et al.*, 2003). All accumulating evidences suggest that, membrane dynamics and their dysfunctions underlie the pathogenesis of a variety of neurodegenerative diseases.

1.4. Axonal Transport

All cells must constantly supply and distribute new materials, such as some organelles, proteins and membranes apart. In the case of motor neurons, this distribution is quite a challenge, due to their very long axons. Thus, MNs need a highly efficient transport system.

The major components of axonal transport (AT) are a group of motor proteins and the cytoskeletal networks of microtubules, actin filaments and neurofilaments. Actin filaments are highly enriched in the cell body, while microtubules and NFs are in the length of the axon (Ström *et al.*, 2008). Microtubules in axons provide stability and polarity, as they are all oriented with a minus end directed to the cell body and a plus end towards the axonal terminal (Joshi, 1993).

Axonal transport is divided into two, according to the speed of movement of transported material. The 'fast axonal transport' term is used for fast-moving materials like membrane-bonded vesicles and organelles such as mitochondria, and the 'slow axonal transport' is the term for mostly polymerized cytoskeletal proteins, which move only a fraction of a millimeter per day (Molecular Cell Biology, 4th edition, 2000).

Cytoplasmic dynein, kinesin and myosin are the motor proteins that use the cytoskeletal networks as tracks, in order to regulate intracellular transport. These motors are structurally similar with a globular domain (the head), forming their motor domain, and a rod-shaped domain (the tail), involved in cargo binding. Dyneins and kinesins contain a microtubule-binding domain in their head domain, whereas myosins generally contain an actin-binding domain. They bind to the cytoskeletal networks through these domains and hydrolyze ATP in order to slide along tracks (Cao *et al.*, 2004; Weber *et al.*, 2004).

1.4.1. Kinesin

The superfamily of kinesin proteins (KIFs) is composed of 14 family members. KIFs are heterotetramers, with two heavy chains, forming the globular motor domain, and two light chains, forming the cargo binding domain. The motor domain is highly conserved,

whereas the cargo binding domain is significantly diverged to enable the ability of binding to a wide range of cargoes. Kinesin-family member 3A (KIF3A) has been identified to be a protein binding subunit of KIFs (Camargo *et al.*, 2007), whereas kinesin-family member 3B and 5A (KIF3B, KIF5A) are linked with motor activity (Nonaka *et al.*, 1998; Niclas *et al.*, 1994). Although the majority of KIFs are involved in the anterograde direction of transport (toward the plus end), a few of them have been shown to be active in opposite direction (Figure 1.6.). These anomalous KIFs regulate microtubule dynamics rather than taking a role in transport (Miki *et al.*, 2001). KIFs are the major motor proteins involved in anterograde fast AT of various membrane-bounded organelles, including mitochondria, synaptic vesicles and axolemal precursors (Lepold *et al.*, 1992)

1.4.2. Dynein and Dynactin

Cytoplasmic dynein is a large complex consisting of two heavy, two intermediate, four light intermediate and various light chain structures and multiple accessory proteins. The dimeric dynein heavy chain contains the motor domain and a flexible stalk that interacts with other dynein subunits. The auxiliary subunits, that bind to the tail, provide the binding diversity of dynein (Pfister *et al.*, 2006).

In vivo, dynein needs to complex with dynactin (DCTN) in order to operate most of its functions. Dynactin increases the motor efficiency and serves as an adaptor between dynein and various cargoes (Schroer *et al.*, 2004). The DCTN assembly has multiple subunits. A filament base, formed by actin-related protein and a projecting sidearm linked to the base by a shoulder domain form its distinct structure. The projecting sidearm of DCTN is composed of two dimeric p150 ^{Glued} subunits (DCTN1) (Melkonian *et al.*, 2007). The p27 (DCTN2) and p22 subunits (DCTN3) have a role in protein binding ability of dynein (Berrueta *et al.*, 1999; Lehner *et al.*, 2004). The dynein/dynactin complex is involved in retrograde (toward minus end) fast AT (Figure 1.6.).

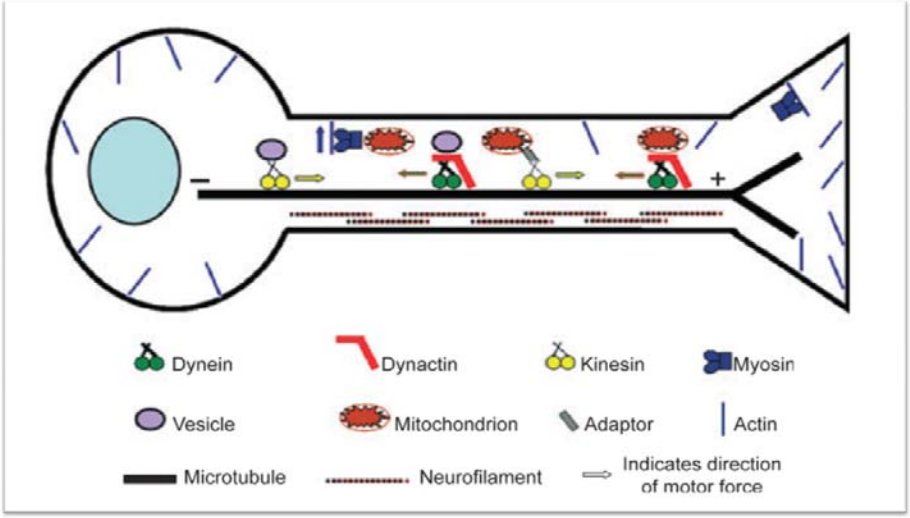

Figure 1.6. Axonal transport processed by the motor proteins and cytoskeletal networks.

1.4.3. Regulatory Mechanisms

Studies of axonal transport show that dyneins and kinesins can function in a dependent manner, such that, the movement in one direction would affect the movement in the opposite direction (Brady *et al.*, 1990; Martin *et al.*, 1999). Two major proposed models for explaining the bi-directionality and interdependency of cargo transport are, 'tug-of-war' and 'coordinated' models. In the 'tug-of-war' model, anterograde and retrograde transport of the cargo is performed simultaneously, and the direction of overall transport is determined by the dominant motor proteins' direction at a given time. However, in the coordinated model, either anterograde or retrograde transport is performed by the certain activation of the KIFs or dyneins. This activation of motor proteins can be enhanced by direct interactions of several adaptor proteins. The microtubule associated proteins, such as tau, are shown to be examples of these adaptor proteins (Dixit *et al.*, 2008). The slow transport of NFs is suggested to be in a 'tug-of-war' manner, while peroxisomes and vesicles are suggested to be in a coordinated manner (Gross *et al.*, 2002; Holzbaur *et al.*, 2004).

The need to deliver selected materials to the axon terminals implies the existence of a targeting mechanism in axonal transport. As a result of several studies by different groups, phosphorylation-dependent intracellular signaling mechanisms have been proposed in coordinating and regulating fast AT (Morfini *et al.*, 2009). Biochemical, pharmacological

and cell biological experiments identified multiple kinases that regulate motor proteins (Brill and Pfister, 2000). These kinases differentially regulate enzymatic and non-enzymatic properties of the motor neurons by modifying, directly or indirectly, specific subunits. For instance, the microtubule-binding, cargo-binding and ATPase activities, are modified by the phosphorylation of the specific subunits (Morfini *et al.*, 2009; Pigino *et al.*, 2009).

1.4.4. Axonal Transport and Motor Neuron Disease

As mentioned before, a highly efficient axonal transport system is essential for the proper functioning and health of the motor neurons. Thus, any defects in the microtubule assembly or the motor proteins can have severe effects on these cells.

Impaired motor dynamics is implicated in several motor neuron diseases. Spastin in HSP is thought to be a microtubule-severing protein involved in axon development (Hazan *et al.*, 1999) The 50 per cent decrease in spastin protein impairs axonal maintenance (Wood *et al.*, 2006). Although the exact function of the other major HSP-linked gene, atlastin is unknown, it has been suggested to be involved in vesicle trafficking events (Zhao *et al.*, 2001). Finally KIF5A has been implicated in HSP (Reid *et al.*, 2002). Several studies have highlighted axonal transport defects as major contributing factors to ALS. As discussed before, alsin has been strongly related to intracellular trafficking. Indeed, defects in axonal transport are one of the earliest pathologies observed in mutant SOD1 mice (Williamson and Cleveland, 1999). Recent findings of mutant SOD1 and dynein interactions are suggested along with several mechanisms that can explain the role of mutant SOD1 in axonal transport; these are physical blockage of dynein by its aggregates, disruption of microtubule formation and dynein/dynactin complex integrity, disturbance of dynein motor activity and masking of cargo binding sites (Ström *et al.*, 2008).

All genes responsible for motor neuron diseases are expressed both in neurons and nonneuronal cells, but the effect of their mutations selectively target MNs. These mutations might disrupt the intracellular transport process of a motor neuron-specific cargo, or if the cargo is not MN-specific, the MNs might be more susceptible to any disturbance in the transport of that cargo, due to their larger volumes.

2. PURPOSE

Mutations in alsin, leading to loss of function of the protein, result in different motor neuron disorders. Although these diseases have distinguished clinical features, they share a common pathogenesis, which is motor neuron degeneration. Thus, it is crucial to unravel the exact function of alsin and the mechanisms it is involved in.

Considering the fact that alsin is a ubiquitously expressed protein, the selective degeneration in motor neurons is very remarkable. The most significant difference of motor neurons from other cell types is the presence of long axons. This indicates a possible involvement of axonal transport defects in disease progression.

In the light of the above evidence, the aim of this study is to understand the function of alsin using two different approaches:

- investigating the effects of mutant alsin on cells with motor neuron characteristics (NSC34 cells), by transient transfection.
- analysing alsin's possible interactions with selected motor proteins (DCTN1, DCTN2, DCTN3, KIF3A, KIF3B and KIF5A) by using qRT-PCR, Immunofluorescence and Immunoprecipitation.

3. MATERIALS

3.1. Cell Lines

3.1.1. Neuroblastoma 2A (N2A)

N2a cells were purchased from ATCC Company, USA.

3.1.2. Neuroblastoma-Spinal cord hybrid cell line (NSC34)

NSC34 cells were kindly provided by Prof. Dr. Maria Teressa Carri from the University of Rome.

3.2. Buffers and Solutions

All solid and liquid chemicals used in this study were purchased from Sigma (USA), unless stated otherwise in the text.

3.2.1. Cell Culture

Table 3.1. Cell culture materials.

0.5 per cent Trypsin-EDTA 1X	:	GibcoBRL, USA
Complete DMEM	:	10 per cent FBS 1 per cent Pen Strep 1 per cent GlutaMAX 1 per cent NEAA

Table 3.1. Cell culture materials (continued).

Complete MEM	:	10 per cent FBS 1 per cent Pen Strep 1 per cent GlutaMAX 1 per cent NEAA
Differentiation MEM	:	1 per cent FBS 1 per cent Pen Strep 1 per cent GlutaMAX 1 per cent NEAA
DMSO	:	AppliChem, Germany
Dulbecco's Modified Eagle Medium (DMEM)	:	GibcoBRL, USA
Fetal Bovine Serum (FBS)	:	GibcoBRL, USA
Freezing Medium	:	25 per cent FBS 1 per cent Pen Strep 1 per cent GlutaMAX 1 per cent NEAA
GlutaMAX I 100X	:	GibcoBRL, USA
G418 antibiotic	:	Sigma, USA
Horse Serum (HS)	:	GibcoBRL, USA
Hygromycin	:	Sigma, USA

Table 3.1. Cell culture materials (continued).

MEM Non-essential amino acid (NEAA) 100x	:	GibcoBRL, USA
Minimum Essential Medium (MEM)	:	GibcoBRL, USA
Opti-MEM I	:	GibcoBRL, USA
Penicillin/Streptomycin (Pen/Strep):		GibcoBRL, USA
Phosphate Buffered Saline (PBS)	:	GibcoBRL, USA
Retinoic Acid	:	Sigma, USA
RNaseZAP	:	Sigma, USA

3.2.2. Transformation and Transfection

Table 3.2. Transformation and transfection materials.

FuGENE®HD Transfection Reagent:		Roche, Germany
Kanamycin	:	Sigma, USA
LB Agar		1Lt LB medium 15g Agar
LB medium (1 Lt)	:	10g Tryptone 5g Yeast Extract 5g NaCl

Table 3.2. Transformation and transfection materials (continued).

Lipofectamine™ LTX with PLUS™ Reagent	:	Invitrogen, USA
Protease Inhibitor	:	Calbiochem, USA

3.2.3. Protein Isolation

Table 3.3. Protein isolation materials.

Cell Lysis Buffer	:	50mM Tris 150mM NaCl 1 per cent Triton-X-100
Protease Inhibitor	:	Roche, Germany
RIPA Buffer	:	50mM Tris pH 7.4 1mM EDTA 5mM $MgCL_2$ 1 per cent Triton 0.25 per cent Na-deoxychol

3.2.4. Western Blot (WB)

Table 3.4. Western blot materials.

Acrylamide:Bisacrylamide	:	29.2 g/100 ml acrylamide 0.8 /100 ml N`N`-bis-methylene-acrylamide
Ammonium Persulfate	:	10 % APS (w/v) in dH2O
Blotto, NonFat Dry Milk	:	Santa Cruz Biotechnologies, USA
Chemiluminescence	:	Santa Cruz, USA

Table 3.4. Western blot materials (continued).

Coomassie Brilliant Blue	:	Santa Cruz, USA
Developer	:	Kodak, USA
Fixer	:	Kodak, USA
Ponceau Staining Solution	:	Qiagen, USA
Protein Marker	:	PageRuler™ Plus Prestained Protein Ladder
Resolving Gel (8%)	:	2.7ml Acryalmide 30% 1.25ml Tris- HCl 3M pH 8.8 100µl SDS 10% 5,9ml H2O 75µl APS 10% 7,5µl TEMED
Running Buffer	:	25mM Tris-HCl, pH 8.3 192mM Glycine 0,1 per cent SDS
Sample Buffer	:	50 mM Tris 50 per cent β-mercaptoethanol 10 per cent Glycerol
SDS	:	Merck, Germany
Stacking Gel (4%)	:	0,53ml Acryalmide 30% 1ml Tris- HCl 0,5M pH 6.8 40µl SDS 10% 2,39ml H2O 40µl APS 10% 4µl TEMED

Table 3.4. Western blot materials (continued).

TBS-TWEEN	:	160mM NaCl 20mM Tris 0.05 per cent TWEEN
Transfer Buffer	:	25mM Tris-Glycine 20 per cent Methanol
TWEEN	:	CalbioChem, Canada

3.2.5. Immunoflourescence (IF)

Table 3.5. Immunoflourescence materials.

Nuclear dye DAPI	:	Santa Cruz Biotech., USA
Paraformaldehyde	:	4 per cent Formaldehyde in PBS pH 7.2-7.4
Triton-X-100	:	CalbioChem, Canada

3.2.6. Immunoprecipitation (IP)

Table 3.6. . Immunoprecipitation materials.

IP Buffer PH 8	:	50mM Tris 150mM NaCl 1 per cent Triton-X-100
IP Matrix	:	Sigma, USA

3.3. Fine Chemicals

3.3.1. shRNA and Vectors

shRNA sequence was designed using the online tool of Ambion Company. It was sent by the same company fused into a pSilencer vector. Wild-type and mutant Alsin constructs, inserted into pCruz GFP vectors, were kindly provided by Dr. İzzet Enünlü from our laboratory.

Table 3.7. . shRNA vectors.

pSilencer™ 2.1-U6 hygro Vector (Figure 3.1.)	:	Ambion, USA
pCruz GFP™ Expression Vector (Figure 3.2.)	:	Santa Cruz Biotechnologies, USA

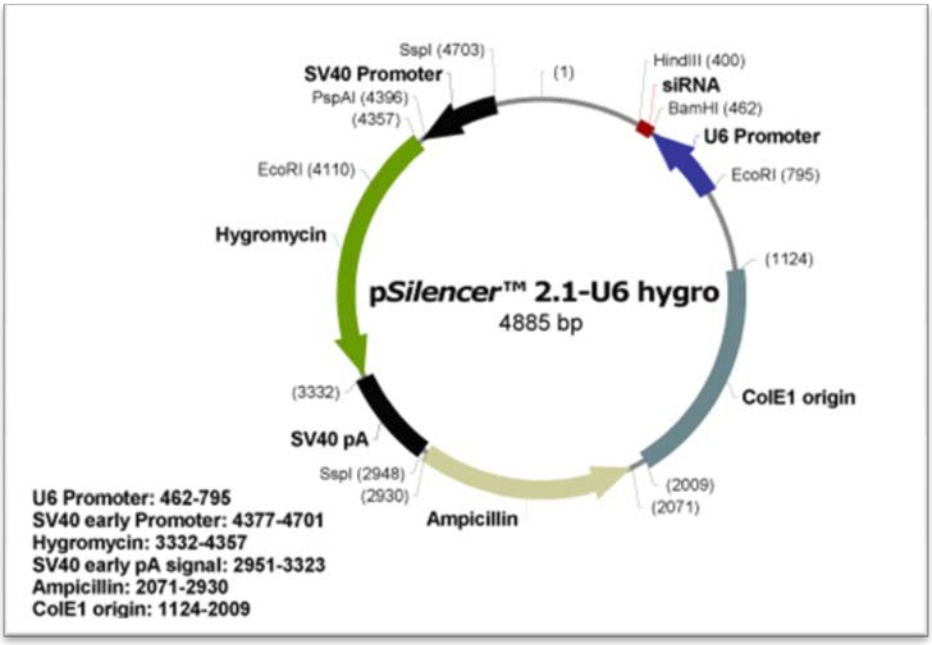

Figure 3.1. Map of the pSilencer™ 2.1-U6 hygro Vector (www.ambion.com).

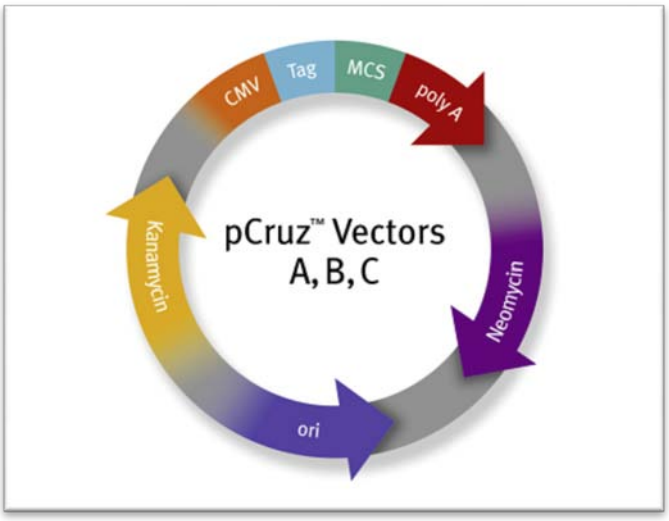

Figure 3.2. Map of the pCruz GFP™ Expression Vector (www.scbt.com).

3.3.2. Primers

Primers used in this study were adopted from Harvard Primer Bank (Table 3.1.)

Table 3.8. Primer sequences of the genes involved in the study.

DCTN 1	**Forward :** 5' CTTTGTACGCCAGTCCCAGAT 3'
	Reverse : 5' CCGTCGAGTTGTGGTCTTTC 3'
DCTN 2	**Forward :** 5' TTGATTTCTCAGATCGCATTGGA 3'
	Reverse : 5' CATGCAGTAGTCGTTGGTACTT 3'
DCTN 3	**Forward :** 5' GCAGGTGGCTTTAGGGAACAT 3'
	Reverse : 5' CAGGTATGGCAATGCGATCAA 3'
KIF3A	**Forward :** 5' ATGCCGATCAATAAGTCGGAGA 3'
	Reverse : 5' GTTCCCCTCATTTCATCCACG 3'
KIF3B	**Forward :** 5' CGACGCTGTGTATGATTGGAA 3'
	Reverse : 5' GCAAAAATTGTGCCATTGAAACC 3'
KIF5A	**Forward :** 5' ATGGCGGAGACTAACAACGAA 3'
	Reverse : 5' TGGAAAATGGGGATGAACTTGTC 3'
Alsin	**Forward :** 5' TCCAGTTCTTGCTATGAGTCTCT 3'
	Reverse : 5' GGAATCCGTCATTTTCCCAGG 3'
β-Actin	**Forward :** 5' GGCTGTATTCCCCTCCATCG 3'
	Reverse : 5' CCAGTTGGTAACAATGCCATGT 3'

3.3.3. Antibodies

Primary antibodies were used in dilutions shown in Table 3.2.

Table 3.9. Antibodies used in Western Blot analysis.

Antibody	**Source**	**Company**	**Dilution Used**	**Usage**
GFP antibody (ab290)	Goat	Abcam	1:500	WB
beta Actin antibody	Rabbit	Abcam	1:500	WB
Anti-Cytochrome C	Mouse	Abcam	1:500	IF
Dynactin p150 (G-18)	Goat	Santa Cruz BioTech.	1:300	IF, WB
KIF3A (H-155)	Rabbit	Santa Cruz BioTech.	1:300	IF, WB
KIF3B (H-60)	Rabbit	Santa Cruz BioTech.	1:300	IF, WB
Alsin (H-257)	Rabbit	Santa Cruz BioTech.	1:300	IF, WB,IP
Alsin (C-20)	Goat	Santa Cruz BioTech.	1:300	IF, WB,IP

Anti-rabbit lgG, (red and green) : R&D Systems, USA

Anti-goat lgG, (red and green) : R&D Systems, USA

3.4. Kits

3.4.1. qRT-PCR

QuantiTect SYBR Green
RT-PCR Kit : Qiagen, USA

3.4.2. RNA isolation

High Pure RNA Isolation Kit : Roche, Germany

3.4.3. Plasmid Midiprep

QIAfilter Plasmid Midi Kit : Qiagen, USA

3.5. Equipments

Table 3.10. Equipments used in this thesis.

Equipments	Models
Autoclave	Model MAC-601, Eyela, Japan Model ASB260T, Astell, UK
Balances	Model VA124, Gec Avery, UK Model CC081, Gec Avery, UK TE612, Sartorious, Germany
Capillaries	LightCycler®Capillaries (20µl), Roche, Germany
Centrifuges	Allegra X22-R, Beckman Coulter, USA Centrifuge 1-15, Sigma, USA Centrifuge 2-16K, Sigma, USA
Cover Slips	22x22 mm, ISOLAB Laborgeräte, Germany
Deep Freezers	2021D (-20^{0}C), Arçelik, Turkey Sanyo (-70^{0}C), Sanyo, Japan
Electrophoretic Equipments	Minicell Primo E320, Thermo, USA Wide Sub Cell GT BIO-RAD, USA Mini-PROTEAN 3 Cell, BIO-RAD, USA Mini Trans-Blot, BIO-RAD, USA

Table 3.10. Equipments used in this thesis (continued).

Eppendorf Tubes	1,5 ml Boil-Proof Microtubes, Axygen, USA 0,2 ml and 0,5 ml Thin Wall Flat Cap PCR tubes, Axygen, USA
Falcon Tubes	EasyOpen 50 ml Centrifuge Tubes, JET BIOFIL, USA
Films	Kodak, USA
Flasks	Cell Culture Flask, 50 ml, 690160, GREINER BIO-ONE, Germany Cell Culture Flask, 250 ml, 658170, GREINER BIO-ONE, Germany
Gel loading tips	Invitrogen, USA
Haemocytometer	MARIENFELD, Germany
Heat Blocks	BBA1, Grant Boekel, UK
Hood	IP44/I, Wesemann, German Class II Biohazard Safety Cabinet, ESCO, USA
Incubator	Forma Series II Water Jacketed CO_2 Incubator Thermo Scientific, USA Hybex Microsample Incubator, SciGene,USA
Kodak, X-ray Processor	Kodak, USA

Table 3.10. Equipments used in this thesis (continued).

Magnetic Stirrer	Chiltern Hotplate Magnetic Stirrer, HS31, UK Hotplates MR3001, Heidolph, Germany
Microscope	DMI4000 B, Leica Microsystems, Germany
Microscope slides	Thermo Scientific, USA
Nitrocellulose Membrane	Nitrocellulose Pure Transfer Membrane (0,22 µm), Santa Cruz Biotechnologies, USA
pH Meter	PB-11, Sartorious, Germany
Pipettes	Pipetus, Hirschmann, Laborgeräte, Germany Pipetman, Gilson, USA 5-10-25 ml Serological Pipettes, GREINER BIO-ONE, Germany
Plates	6-well Tissue Culture Plates, BIOFIL, USA 12-well Cell Culture Plates, GREINER BIO-ONE, Germany
Power Supplies	Constant Power Supply, Pharmacia, Sweden EC250-90 Compact Power Supply, Thermo Scientific, USA ECPS 3000/150 Model 200, BRL, USA Powerpac 1000, BIO-RAD, USA

Table 3.10. Equipments used in this thesis (continued).

Real-Time PCR Systems	LightCycler 2.0 System, Roche, Germany
Refrigerator	4250T, Arçelik, Turkey
Reservoir	Corning Incorporated Costar, USA
Shaker	Duomax 1030 Platform Shaker, Heidolph, Germany Reax 2 Overhead Shaker, Heidolph, Germany
Spectrophotometer	NanoDrop ND-1000, Thermo, USA
Electrophoresis Tank	Mini Protean 3 Cell, BIO-RAD, USA
Thermocyclers	C312, Techne, UK iCycler, BIO-RAD, USA Techgene, Progene, UK Touchgene Gradient, Progene, UK
Tips	1000µl, 200µl, 100µl, 10µl Universal Fit Filter Tips, Axygen, USA
Vortex	Fisons WhirliMixer, UK Reax Top, Heidolph, Germany
Water Bath	Gemo DT104, TEST Laboratuvar Cihazları, Turkey
Water Purification	Millipore, USA

4. METHODS

4.1. Cell Culture

4.1.1. Maintenance of the N2a and NCS34 Cell Lines

N2a cells were maintained in complete MEM and NCS34 cells in complete DMEM in culture flasks at a density of 2-5x100,000 cells/ml, in a humid 37°C incubator, supplied with 5 per cent CO_2. In order to separate the adherent cells, they were rinsed with sterile PBS and trypsinized in 500 µl, 0.05 per cent Trypsin/EDTA for 3 minutes. After detaching the cells, trypsin was inhibited with 5 ml complete medium, and the cells were centrifuged at 1400 rpm for four minutes. Cell pellets were resuspended in complete medium and splitted 1/10.

4.1.2. Cell Counting

Cell suspensions were counted by using a haemocytometer. When the cover slip was placed onto the haemocytometer, five squares of one mm^2, each containing 16 squares become visible under the light microscope. The number of cells in each of the five squares were counted and divided into five, to obtain the average amount of cells present in 1 ml as a factor of 10^4.

4.1.3. Cell Storage

A flask of cells were collected by centrifugation at 1400 rpm for 4 minutes. The cell pellet was resuspended in 5 ml of freezing medium with 10 per cent DMSO. The cells were aliquoted in 1 ml fractions in cryovials and were cooled down to -20°C for an hour before being placed in the -80°C freezer. When needed, cells were defrosted at room temperature and placed in flasks containing pre-warmed complete medium.

4.2. Transformation and Plasmid Midi-prep

Two pCruz GFP™ Expression Vectors, including either a GFP-tagged wild-type (wt) gene or a mutant (mt) Alsin gene that causes early truncation of the protein, were used for transient transfection of NSC34 cells, and empty pCruz GFP™ vector expressing only the GFP protein was used as a control. The sequences of the insertions were checked by using vector specific primers and automated DNA sequencing.

In the transformation experiment, first, 100 µl of competent cells (E.coli, placed in calcium chloride solution) were mixed with 1 ng of plasmid DNA and treated in the following order; on ice for 30 minutes, in 42°C water bath for 1 minute and again on ice for 5 minutes. The bacteria containing the plasmid were grown in antibiotic-free liquid LB medium for 1 hour at 37°C, in order to gain antibiotic resistance. Next, the bacteria were plated on a 100mg/ml Kanamycin-containing LB agar plate and incubated overnight at 37°C. On the next day, one colony from the plate was selected and grown in 30 ml of 100 mg/ml Kanamycin-containing liquid LB medium for 2 hours at 37°C.

QIAfilter Plasmid Midi Kit was used for purification of plasmid DNA. The kit combines QIAfilter Midi cartridges to separate bacterial lysates by filtration. Concentration of the eluted plasmid DNA was determined by the NanoDrop Spectrophotometer. The plasmid DNA was stored at -20°C for further applications.

4.3. Transfection and Generation of the Stable Cell Lines

Commercially purchased mammalian expression vector pSilencer™ 2.1-U6 hygro, including the shRNA sequence, designed identical to the DH/PH domain of the Alsin gene, was used in 2µg concentration for knocking-down experiments. A pCruz GFP™ Expression Vector was used both for introducing the full-length alsin into the N2a cells for generating an alsin overexpressing stable cell line, and transient transfection studies of NSC34 cells with the wt and mt alsins. Transfection reagent (FuGENE®HD Transfection Reagent for N2a cells and Lipofectamine™ LTX with PLUS™ Reagent for NSC34 cells) and expression vector were used in 8: 2 (V/W) ratio and incubated 15 minutes at room temperature. During this period, the complete medium of the cells were removed and

washed with sterile PBS in order to take away the protein leftovers. Discarded medium was replaced by antibiotic and serum-free Opti-MEM, then the vector-reagent complex was added to the medium. Finally, N2a cells were placed into the incubator for 24 hours and NSC34 cells for 5 hours, before changing Opti-MEM with the complete medium. Desired expression of the vector was obtained after 48-72 hours of incubation in N2a, and 24 hours of incubation in NCS34 cells.

In the hygromycin titration experiments, cells were seeded in a 12-well plate at 60 per cent confluency. After an overnight incubation, cells were exposed to different hygromycin concentrations to find out the optimum deadly concentration, which will be used in stable transfection experiments. Antibiotic containing medium was refreshed every three days and at the end of the tenth day, optimum hygromycin concentration was determined. Previously transfected cells expressing the desired vector were exposed to the optimum hygromycin concentration. Since the vector had the hygromycin resistance gene, cells that expressed the vector resisted the antibiotic, whereas the others died.

4.4. Protease Inhibition

Protease inhibition was performed in NSC34 cells, transfected with wt- and mt-alsins. After transfecting cells with the plasmid DNA 5 µM CalbioChem's Protease inhibitor was added to the environment and mixed well gently. Cells were incubated at the humid 37°C incubator and were collected after 24 hours.

4.5. RNA Isolation

RNA was always handled with equipments previously wiped with RNase inhibitor. Isolation was performed using Roche High Pure RNA Tissue Kit.

The growth medium was discarded and the cells were detached by 500 µl of sterile PBS. Two hundred µl of cells were transferred to Eppendorf tubes and mixed with 400 µl Lysis/-Binding Buffer and vortexed for 15 seconds. Next, the lysate was transferred to High Pure Filter Tubes and centrifuged 15 seconds at 8000 x g. Master DNase mix, containing 90 µl DNase incubation buffer and 10 µl DNase working solution applied onto

the filters of the High Pure Filter Tubes, followed by incubation at 20^{o}C for 15 minutes. Then, 500 µl of Wash Buffer I was added, which was centrifuged for 15 seconds at 8,000 x g; the flow-through was discarded. In the second wash, 500 µl of Wash Buffer II was added, followed by centrifugation for 15 seconds at 8,000 x g; again the flow-through was discarded. In the last wash, 300 µl of wash buffer II was added, followed by centrifugation for 2 minutes at 13,000 x g. The filter was carefully removed and put into a sterile Eppendorf tube. Finally, 50 µl Elution Buffer was applied to the filter and centrifuged for 1 minute at 8,000 x g. Concentration of the eluted RNA was determined by the NanoDrop Spectrophotometer and it was stored at -80^{o}C for further use.

4.6. Quantitative Real-Time PCR Experiments (qRT-PCR)

DCTN1, DCTN2, DCTN3, KIF3A, KIF3B and KIF5A, the motor protein coding genes, were selected to be investigated as interacting partners of Alsin. These genes were selected, because they are subunits of the motor protein complex. Besides, DCTN1, DCTN2 and KIF5A were chosen since they are linked to neurodegenerative diseases as opposed to DCTN3, KIF3A and KIF3B which are not associated with any neurodegenerative disorder. qRT-PCR experiments were performed for these genes by using the Qiagen, QuantiTect SYBR Green RT-PCR Kit. SYBR Green is a fluorogenic dye that exhibits little fluorescence when in solution, but a strong signal when binding to double-stranded DNA. Thus, as the PCR product accumulates, fluorescence increases and this increase is converted to a graph by the computer. In this experiment β-Actin was used as reference gene and RNAs isolated from the cells were used as samples to construct the curve.

For each reaction capillary, 2 µg RNA was mixed with 20 pmol forward and reverse primers, 10 µl of 2X SYBR PCR Master Mix and 0.2 µl of QT-Mix was added, then the total volume was completed to 20 µl by PCR-grade water. Next, capillary tubes were inserted to the LightCycler and their information was entered into the LightCycler software system (Version 4.0). Absolute quantification option was chosen. The following PCR conditions were applied as shown in Table 4.1.

In each experimental set, the target and the reference gene, β-Actin were analysed in three replicas and the average of the replicas were used in further data analysis. ‘Concentration of target gene/concentration of reference gene’ was accepted as normalized data and transferred to Microsoft Excel to be illustrated as graphs. The online tool for Student’s t-Test was used in order to analyse the statistical significance of the data sets.

Table 4.1. q-RT PCR conditions.

Reverse Transcription:	50°C	20 min.	
Initial Denaturation:	95°C	15 min.	
Denaturation:	95°C	15 sec.	45 cycles
Annealing:	60°C	20 sec.	
Extension:	72°C	30 sec.	
Melting:	65°C	15 sec.	
Cooling:	40°C	30 sec.	

4.7. Western Blot Analysis

Three out of six selected genes, DCTN1, KIF3A and KIF3B, were further analysed at protein level by Western Blot Analysis, actin was used as control.

4.7.1 Sample Preparation

Cells were washed and collected with cold PBS into the Eppendorf tubes, then centrifuged at 4°C 1500 rpm for 5 minutes. The supernatant was removed and the pellet was washed with 100 µl of protease inhibitor, cell lysis buffer for N2a and RIPA Buffer for NSC34 cells were added. Cell lysis buffer protocol was followed by rotating the tubes at 4°C for 30 minutes. Next the tubes were centrifuged at 4°C 10 000 x g for 15 minutes and the protein containing supernatant was collected to a sterile Eppendorf tube. On the other hand in RIPA buffer protocol, tubes were incubated on ice for 5 minutes before being centrifugated at 4°C 14000 rpm for 10 minutes and then the protein containing supernatant was transferred to a sterile Eppendorf tube. Proteins were stored at -80°C for further use.

4.7.2. Bradford Assay

After preparation of protein lysates, the Bradford assay was performed to determine the protein concentration in the samples. 10 mg/ml stock solution, 1 mg/ml ready-to-use BSA solutions and seven replica standard BSA solutions with different concentrations were prepared from this stock solution (Table 4.2.).

Ready-to-use BioRad Bradford reagent: Quick Start™ Bradford Protein Assay was used for analyses, and lysates were diluted 10: 1. For each standard measurement, 20 µl standard was added to 1 ml of Bradford reagent, and for sample measurement, 2 µl sample and 18 µl water were mixed, which were then added to 1 ml of Bradford solution in an Eppendorf tube. After 5 minutes of incubation period, measurements were taken on a Bio-Rad spectrometer, using the Bradford Assay program.

Table 4.2. Preparation of BSA standards for Bradford Assay

Standard Concentration (mg/ml)	**1 mg/ml BSA (ml)**	**Water (ml)**
0,0	0,0	1,0
0,2	0,2	0,8
0,4	0,4	0,6
0,6	0,6	0,4
0,8	0,8	0,2
1,0	1,0	0
1,2	0,12 (from 10 mg/ml stock)	0,88

After calculating the lysate concentrations, aliquots with equal concentrations were prepared using lysis buffer. 1X sample buffer was added to each aliquot and 25 µg of protein was loaded to gel. The samples were heated to 95^{o}C for 4 minutes for denaturation and then immediately placed on ice before being loaded to the gel.

4.7.3. SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

A single SDS gel was prepared for 10 samples and the Bio-Rad Mini-PROTEAN 3 cell system was used for casting and running of gels. Eight per cent resolving gel was used for actin and the selected target proteins. The concentration of the stacking gel was 4 per cent. Then the gel cassette sandwich was prepared and the resolving gel was poured in it. One ml butanol was added on top of the resolving gel in order to smooth the upper surface and prevent evaporation. Following polymerization, the stacking gel was prepared and poured onto the resolving gel and the comb was inserted. Finally the stacking gel was allowed to polymerize for 20 minutes.

Samples were mixed with a 10X sample buffer in a 9: 1 ratio and boiled for 5 minutes for denaturation. Three µl protein marker and 25 µl sample plus sample buffer mixture were loaded into the gel and ran for 1 hour at 200 V.

Proteins were visualized on the gel by Coomassie Brilliant Blue (CBB) staining. The gel was placed in 1X CBB dye solution for 15-20 min until bands were seen. Destaining was performed first for 20 minutes, then for 1 hour before continuing with blotting.

4.7.4. Blotting

The electrophoretic transfer process was performed using a Bio-Rad Mini Trans-Blot, Electrophoretic Transfer Cell system. The sandwich was prepared to allow the proteins to transfer from the gel to the membrane with the electric current. The fiber pad was wetted with transfer buffer, and then two pieces of equal-sized wet filter paper (Watmann 3mm) were placed on the pad. The gel, wetted in transfer buffer, was placed on filter papers on the minus side of the sandwich and air bubbles were removed carefully. The nitrocellulose membrane, handled always carefully with a forceps, was also placed in the transfer buffer before it was placed on the gel. Again, two pieces of wetted filter paper and fiber pad were placed on the membrane and the sandwich cassette was closed. The cassette was placed in the electrode module. After placing the electrode module into the chamber, the chamber was filled with transfer buffer. To maintain the even ion and temperature distribution in the tank during the blotting process, a magnetic stirrer was

used. Blotting was performed at 220 mA for 2 hours at 4^{o}C. The success of the transfer process was tested by Ponceau Staining Solution.

4.7.5. Hybridization with Primary and Secondary Antibodies

The membranes were stained with corresponding antibodies of selected proteins. For this purpose, the membrane was blocked for 1 hour with 5 per cent (w/v) milk powder in TBST (TBS containing Tween 20). Then it was incubated in primary antibody solution in 2 per cent milk for 2 hours. Excess and unspecific binding was washed off with TBST for 10 minutes (three times). Then, the membrane was incubated with secondary antibody solution in 1 per cent milk for 1 hour. Again, excess and unspecific binding was discarded by washing the membrane three times in TBST for 10 minutes.

4.7.6. Detection of the Blot

After the last washing step, the membrane was incubated with TBS solution for 5 minutes, then incubated for 1 minute with 10 ml chemiluminescence solution, containing 3 µl of H_2O_2. The membrane was placed on a cassette and covered with a thin acetate paper. The luminescence emitted by the membrane was exposed to films, for 30 seconds, 1 minute and 5 minutes.

The film was submerged into the developer solution for 5 seconds, washed with water and plunged into the fixer for 1 minute with a forceps.

4.8. Immunofluorescence

In immunofluorescence studies cells were previously plated on 6-well plates containing cover slips. First, growth medium of the cells were removed and cells were washed with 1 ml of PBS slowly not to detach the cells. Next 1 ml of 4 per cent paraformaldehyde was added on the cover slips. After a 10 minutes incubation period, paraformaldehyde was removed, one ml of 0.4 per cent Triton-X-100 in PBS solution was applied and incubated for 5 minutes. For saturation, 1 ml of 10 per cent HS containing PBS was added and incubated at room temperature for 30 minutes.

Primary antibody was diluted with 2 per cent HS containing PBS in 1: 400 ratio and added on the cover slips. After the primary antibody was incubated at 37^{o}C for 1 hour, cover slips were washed three times with 1 ml PBS carefully. Secondary antibody was also diluted with two per cent HS containing PBS in 1: 800 ratio, added on the cells and incubated for 45 minutes in a dark place. Next, cover slips were washed three times with 1 ml PBS. Finally the nuclear dye was dropped on the cells and the cover slips were put on the slides. The slides were stored in a dark place for further microscope imaging.

Leica Microsystems DMI4000 B microscope and Leica FW4000 software were used for imaging the fluorescence emitted by the cells.

4.9. Immunoprecipitation

One hundred µl of IP matrix was mixed with 800 µl of IP buffer in an Eppendorf tube, and 5 µg Alsin antibody was added to the mixture. Following rotation of the tubes at 4^{o}C for 1 hour, they were centrifuged at 4^{o}C and 2000 rpm for 2 minutes. Next the supernatant was removed very carefully, to avoid contact with any beads at the pellet. The pellet was resuspended with 800 µl of protein sample, previously isolated with cell lysis buffer, and rotated overnight at 4^{o}C. Next day samples were centrifuged at 4^{o}C, 2000 rpm for 2 minutes and the supernatant was removed precisely. Then, the pellet was washed with 800 µl of IP buffer and centrifuged at 4^{o}C, 2000 rpm for 2 minutes. This step was repeated three times in order to wash away the proteins, except Alsin bonded to antibody-coated beads and its interactors.

In the final step, beads were resuspended in 50 µl of sample buffer and Western Blot analyses were performed.

5. RESULTS

5.1. Effects of Mutant Alsin on Motor Neurons

5.1.1. Design of the Experiment Groups

NSC34 cells, the fusion of mouse N18TG2 neuroblastoma and embryonic spinal-cord cells, were used in this study, because they are easily transfected and they show a multipolar neuron-like phenotype even when they are undifferentiated (Figure 5.1.). Three experiment groups (EG1, 2, 3) with NSC34 cells were designed. The control group was transfected with the GFP-expressing pCruz GFP™ expression vector (EG1) and; the other two groups were separately transfected with wt-alsin- and mt-alsin-inserted pCruz GFP™ expression vectors (EG2 and EG3, respectively). The aim was to determine the presence of any phenotypical defects or abnormalities in the mt-alsin group (EG3), as compared to control (cnt) and wt-alsin groups.

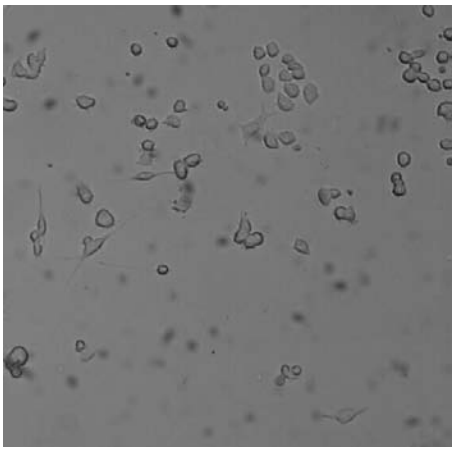

Figure 5.1. Undifferentiated NSC34 cells

5.1.2. Transfection of the NSC34 Cells

5.1.2.1. Transfection Efficiency: The transfection efficiency was determined after 24 hours by GFP fluorescence emitted by the transfected cells. The GFP fluorescence

obtained from the cnt cells was brighter; the transfection efficiency was almost twice as high as the wt-alsin, and five times higher than the mt-alsin transfected cells (Figure 5.2.).

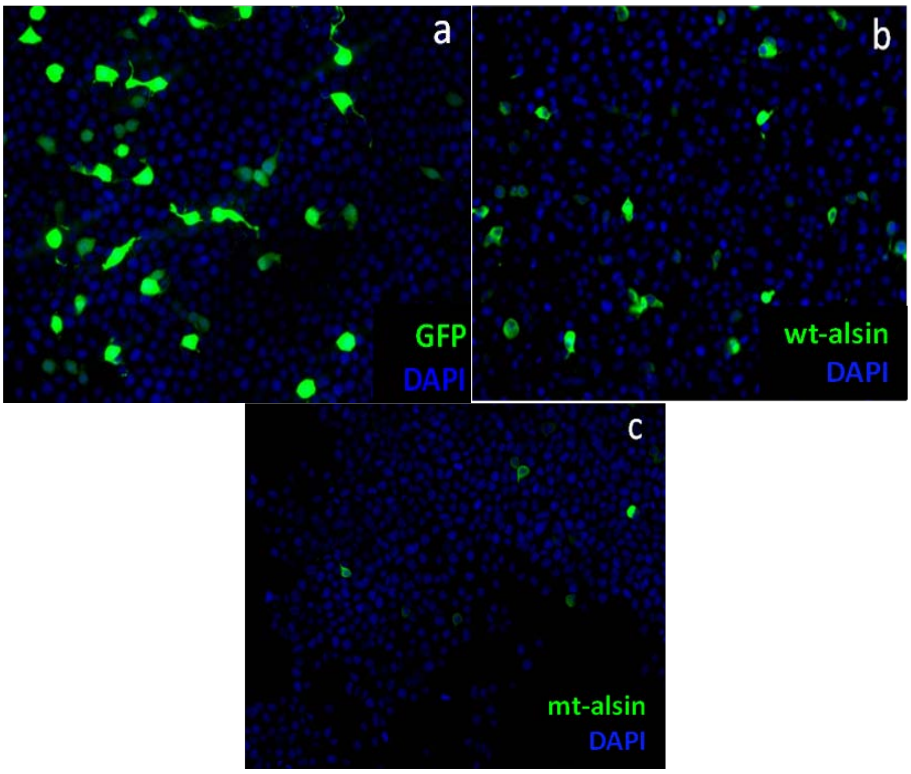

Figure 5.2. Transfection efficiencies of GFP fluorescence in NSC34 cells, 24 hours after transfection: a. EG1: 20 per cent; b. EG2: 12 per cent; c.EG3: 4 per cent.

A decrease in transfection efficiency was observed in all three experiment groups after 48 hours. Especially in mt-alsin-transfected cells a drastic decline was detected as the incubation period was extended (Figure 5.3.).

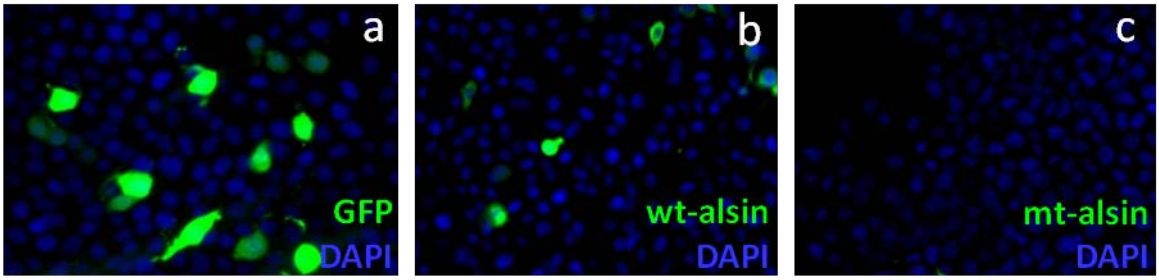

Figure 5.3. Transfection efficiencies of GFP fluorescence in NSC34 cells, 48 hours after transfection: a. EG1: 12 per cent; b. EG2: 5 per cent; c.EG3: 0.1 per cent.

5.1.2.2. Phenotype Detection: The three phenotypic features chosen to be examined in this study were:

- cell viability
- defects in axons
- mitochondrial disorganization

Mutant-alsin-transfected cells showed no difference in the above three characteristics, compared to the cnt and wt-alsin-transfected cells (Figure 5.4.). However, the GFP fluorescence emitted by the mt-alsin protein implied an aggregate-like structure (Figure 5.4.c.).

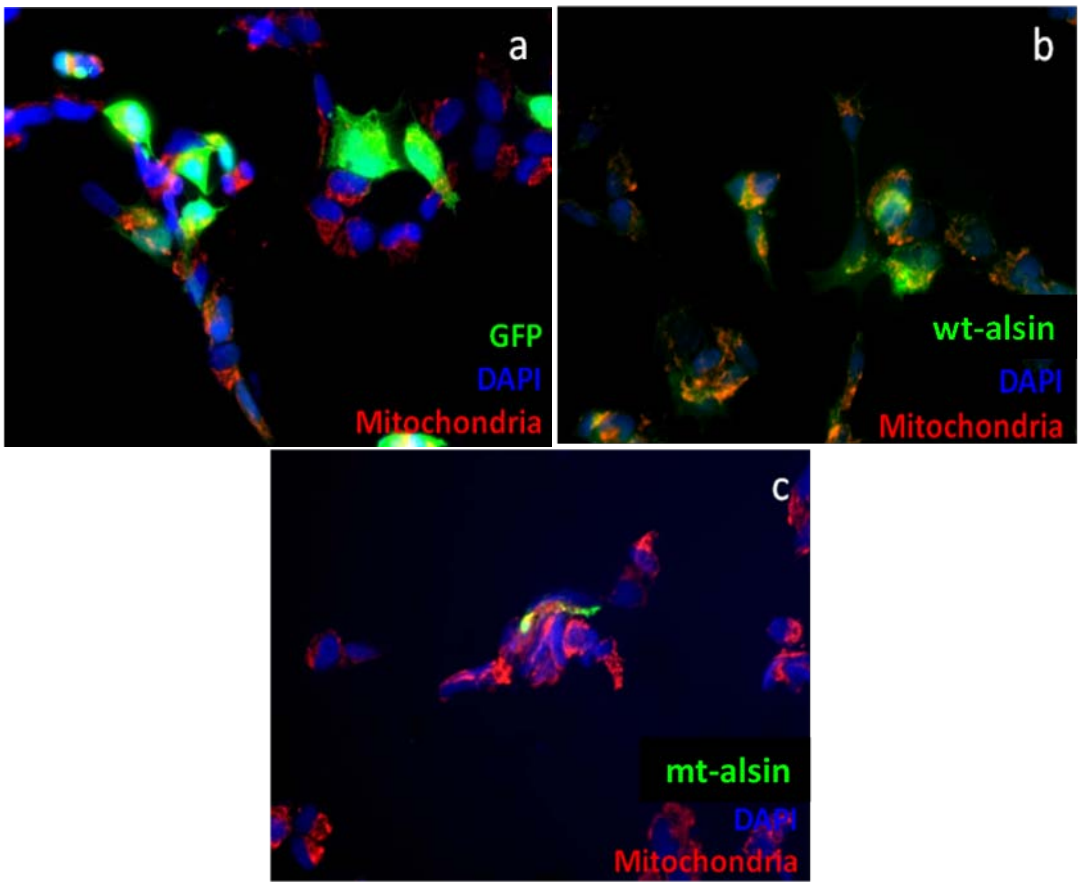

Figure 5.4. Mitochondrial organization and GFP fluorescence of the NSC34 cells, 24 hours after transfection: a. EG1; b. EG2; c. EG3.

5.1.3. Mt-Alsin Aggregation?

5.1.3.1. Western Blot Analysis: In order to confirm the mt-alsin aggregation, Western Blot analysis was performed with 20 mg protein isolated from the NSC34 cells, 24 hours after transfection. The Bradford assay was applied to determine the protein concentration in each sample. A GFP antibody was used to detect the control GFP and the GFP-tagged alsins, while an actin antibody was used as an internal control for the protein levels. The GFP in the control plasmid was a 27 kDa protein. Since alsin was GFP-tagged, the expected wt-alsin band was approximately 210 kDa (184+27 kDa). Mutation in the mt-alsin resulted in the truncation of one third of the protein. Thus, the expected band was approximately 150 kDa, but it presented as a smear. The cnt and wt-alsin bands were observed as expected (Figure 5.5.).

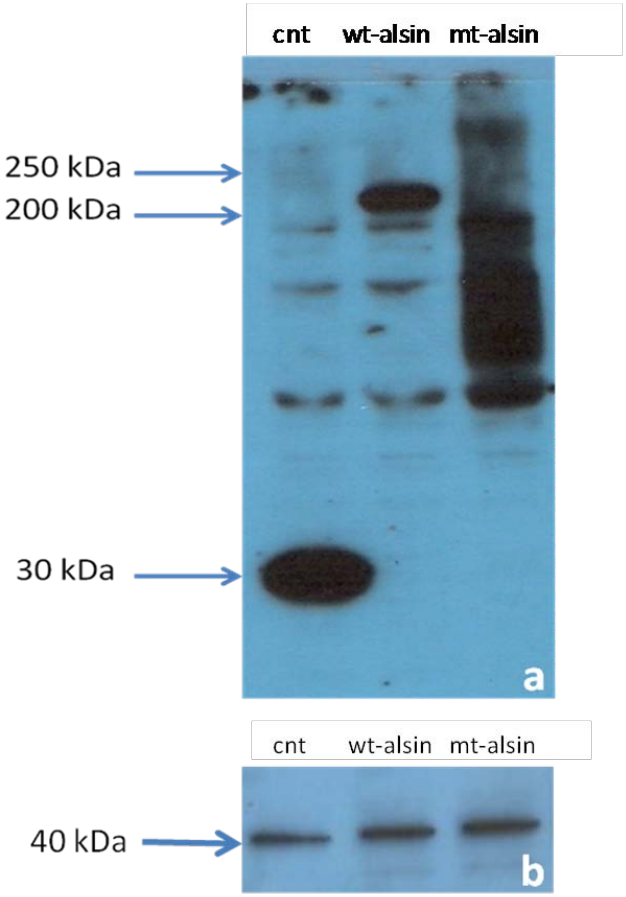

Figure 5.5. Western Blot results of NCS34 cells, transfected for 24 hours: a. GFP in the control plasmid detected at 30 kDa, GFP-tagged wt-alsin detected at 210 kDa and mt-alsin detected as a smear; b. Actin proteins of the transfected NSC34 cells detected at 40 kDa.

Cells, transfected with cnt, wt-alsin and mt-alsin and incubated for 48 hours were further analysed by WB to confirm the decreased amounts of proteins, that IF experiments had indicated. Supporting the results of IF experiments, decrease in the amounts of cnt-GFP and wt-alsin proteins were observed, whereas no mt-alsin protein was detected (Figure 5.6.)

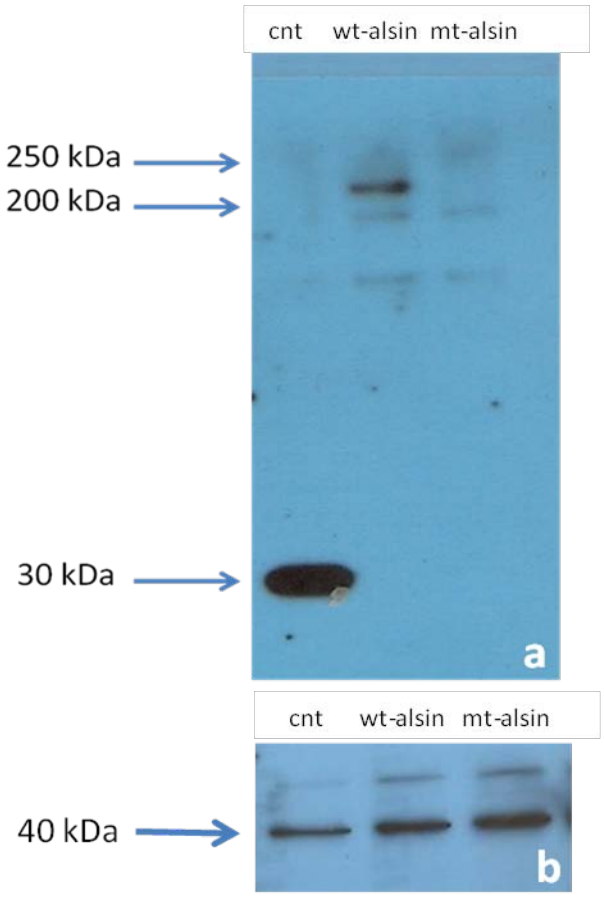

Figure 5.6. Western Blot results of NCS34 cells transfected for 48 hours: a. GFP in the control plasmid at 30 kDa, GFP-tagged wt-alsin at 210 kDa and mt-alsin absent; b. Actin proteins of the transfected NSC34 cells at 40 kDa.

5.1.3.2. Protease Inhibition: Since the IF and WB results indicated a rapid degradation of the mt-alsin, a protease inhibition step was performed as further confirmation. In this step, 5µM of CalbioChem protease inhibitor was added to the plates after the termination of the transfection, and they were incubated for 24 hours.

In control cells, no significant change was detected. However, in wt- and mt-alsins, more green cells were observed and the green signal intensity was increased. WB analysis performed with the protein lysates of these protease-inhibited transfected cells were compared with the proteins of the protease-uninhibited transfected cells (Figure 5.7).In protease-inhibited cells, GFP in the control plasmid was detected at 30 kDa, and GFP-tagged wt- and mt-alsins between 210 and 200 kDa (Figure 5.7. a). In uninhibited transfected cells, GFP in the control plasmid was detected at 30 kDa whereas no wt- and mt-alsin bands were observed (Figure 5.7. c). Actin was used as a control for protein levels (Figure 5.7. b and d).

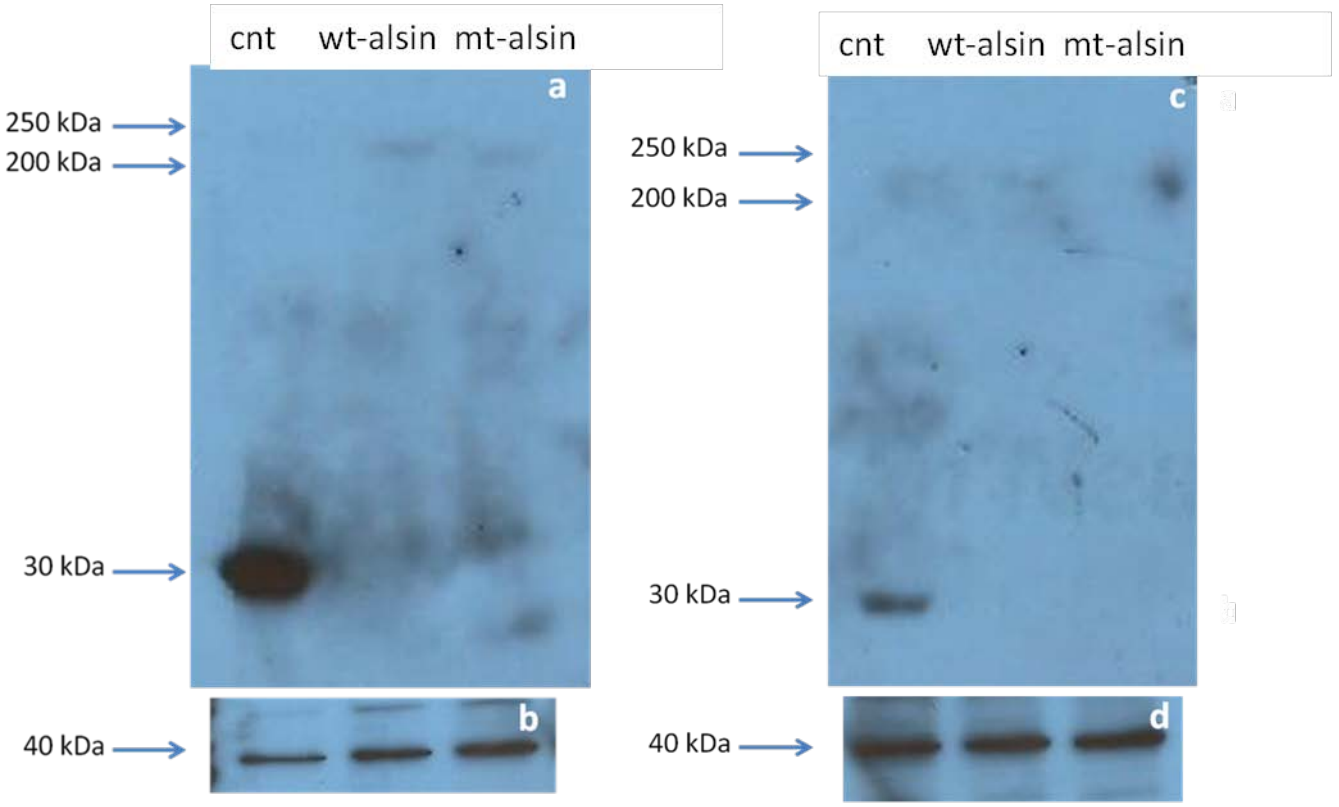

Figure 5.7. Comparison of protein amounts of the protease-inhibited and uninhibited transfected cells: a. protease-inhibited cells; b. actin proteins of experiment a; c. protease-uninhibited transfected cells; d. actin proteins of experiment c.

5.2. Possible Interactions of Alsin with Motor Proteins

5.2.1. Differentiation of N2a Cells

N2a cells, isolated from the brain tissue of albino mice, have neuron-specific characteristics and they can be differentiated easily. In this study N2a cells were differentiated by serum deprivation (Figure 5.8.).

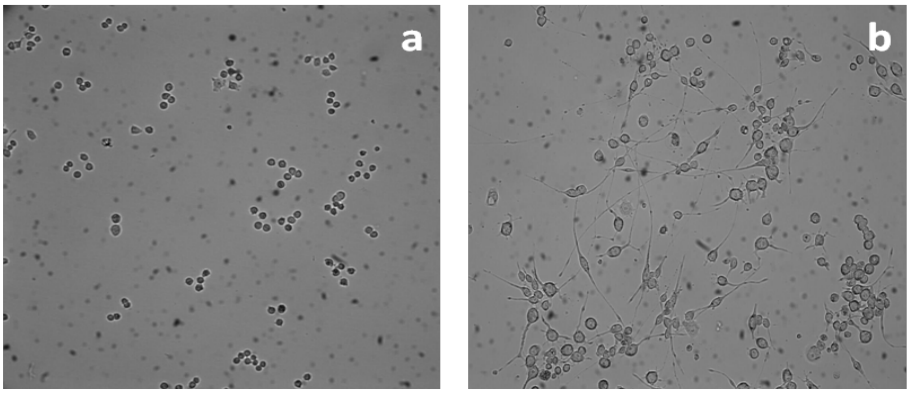

Figure 5.8. a. Undifferentiated and b. differentiated N2a cells.

5.2.2. Generation of Alsin Knock Down Stable Cell Lines

An alsin knock down (kd) stable cell line (alsin-kd) was generated with N2a cells and the kd efficiency was measured by q-RT PCR. The results were normalized with β-actin and compared with wt-N2a control cells (alsin-cnt).

As the first step in investigating the possible interactions of alsin with motor proteins, changes in the expression levels of the selected proteins were detected by q-RT PCR in alsin-kd cells. These results were also normalized with β-actin and compared with the expression levels of the proteins in alsin-cnt cells. As a result, a 73 per cent decrease in expression of alsin was observed in alsin knock-downed cells (Figure 5.9.). The p-value derived from Student's t-Test was <0.0001 referring to extremely significant results.

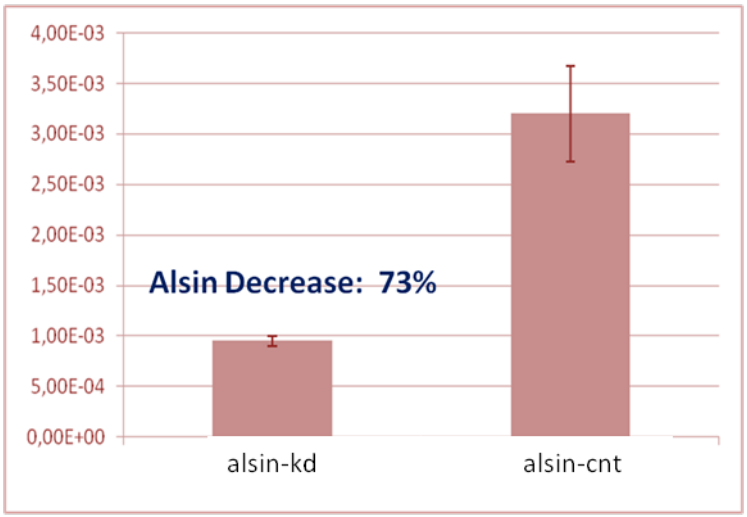

Figure 5.9. Alsin knock-down levels of the stable cell lines (p-value <0.0001).

5.2.3. Quantitative Real-Time PCR Analyses

Six motor proteins are selected upon the impaired motor dynamics implicated in several motor neuron diseases. These are DCTN1, DCTN2, DCTN3, KIF3A, KIF3B, KIF5A. Only three of these proteins showed extremely significant changes in their expression levels with p-values of <0.0001 in alsin-kd stable cell lines: DCTN1 and KIF3A displayed an increase (109 per cent and 17.3 per cent, respectively,), KIF3B on the other hand, showed a decrease of 19.2 per cent with a p-value of 0.0089, referring to a very significant result (Figures 5.10. to 5.12.).

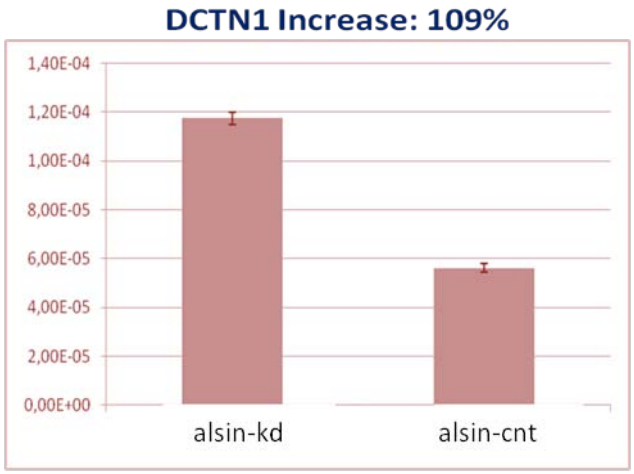

Figure 5.10. A 109 per cent significant increase in expression levels of DCTN1 gene in the alsin-kd stable cell lines (p-value <0.0001).

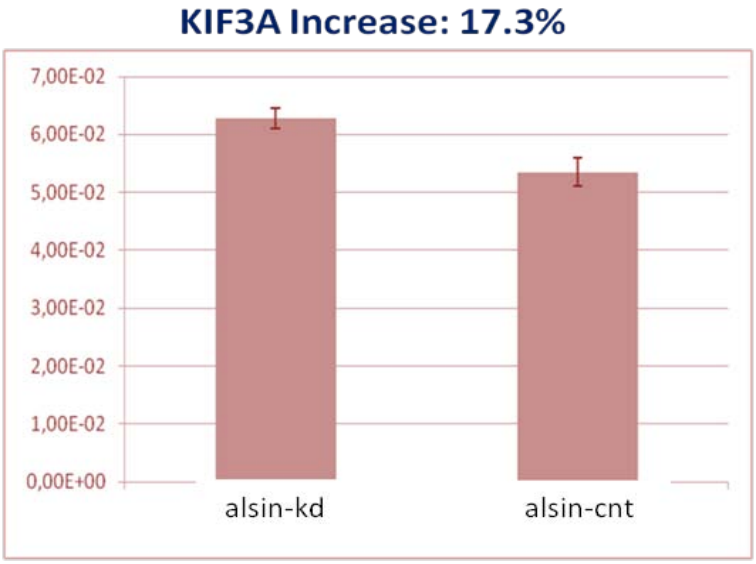

Figure 5.11. A 17.3 per cent significant increase in expression levels of KIF3A gene in the alsin-kd stable cell lines (p-value <0.0001).

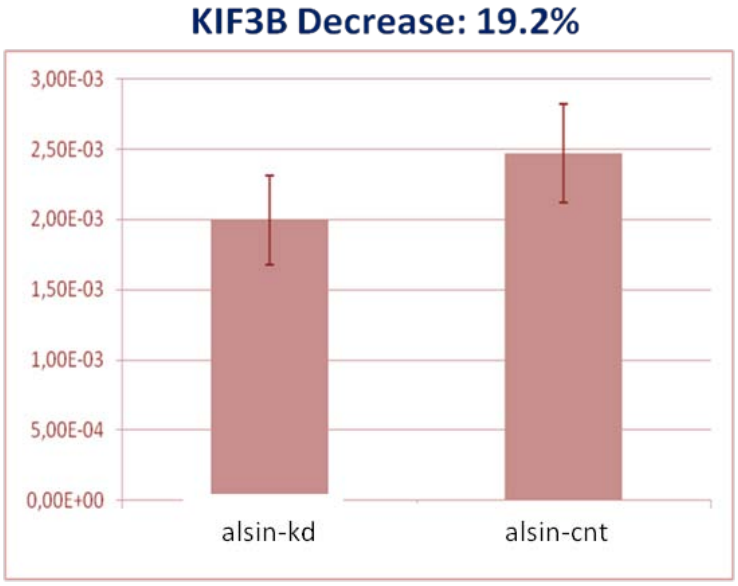

Figure 5.12. A 19.2 per cent significant decrease in expression levels of KIF3B gene in the alsin-kd stable cell lines (p-value 0.0089).

The remaining three genes showed insignificant changes in their expression levels and were excluded from further experiments (Figures 5.13. to 5.15.).

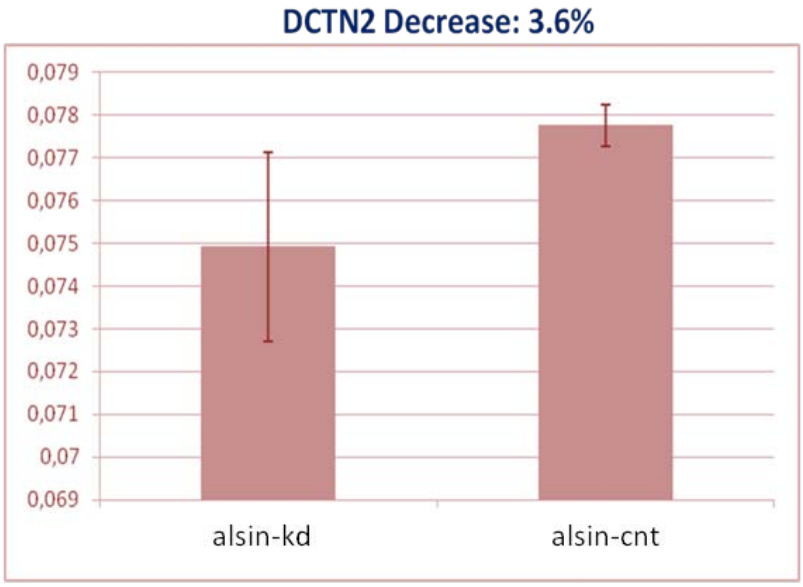

Figure 5.13. A 3.6 per cent increase in expression levels of DCTN2 gene in the alsin-kd stable cell lines is insignificant.

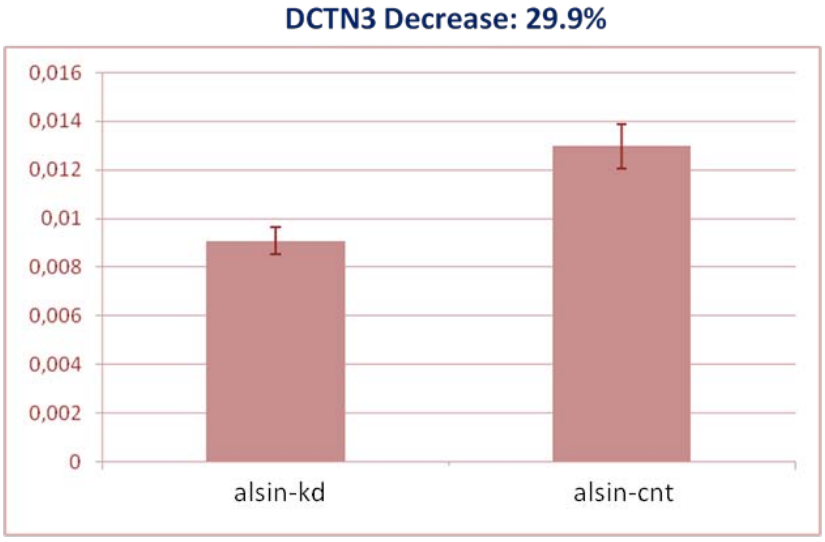

Figure 5.14. A 29.9 per cent increase in expression levels of DCTN3 gene in the alsin-kd stable cell lines is insignificant.

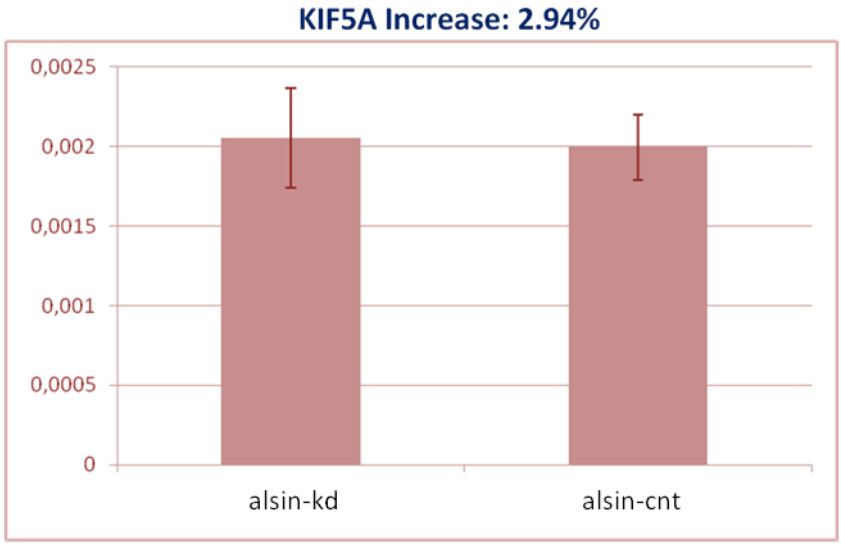

Figure 5.15. A 2.94 per cent decrease in expression levels of KIF5A gene in the alsin-kd stable cell lines is insignificant.

5.2.4. Immunofluorescence Analyses

Immunofluorescence studies were performed in order to localize the three proteins with significant changes in their expression levels, when alsin was knocked down. Alsin was stained red and DCTN1, KIF3A and KIF3B were separately stained green in differentiated N2a cells. The composite images of DCTN1 and KIF3B displayed separate localizations with alsin, whereas KIF3A showed an obvious co-localization with alsin in the perinuclear region, where alsin protein is known to be mostly present (Figure 5.16.).

5.2.5 Immunoprecipitation Analyses

The possible interactions of alsin with DCTN1, KIF3A and KIF3B was further analysed by immunoprecipitation studies. In this approach, beads of the IP matrix were coated with alsin antibody and WB analyses were performed with motor protein antibodies. However, no specific bands were detected in IP experiments.

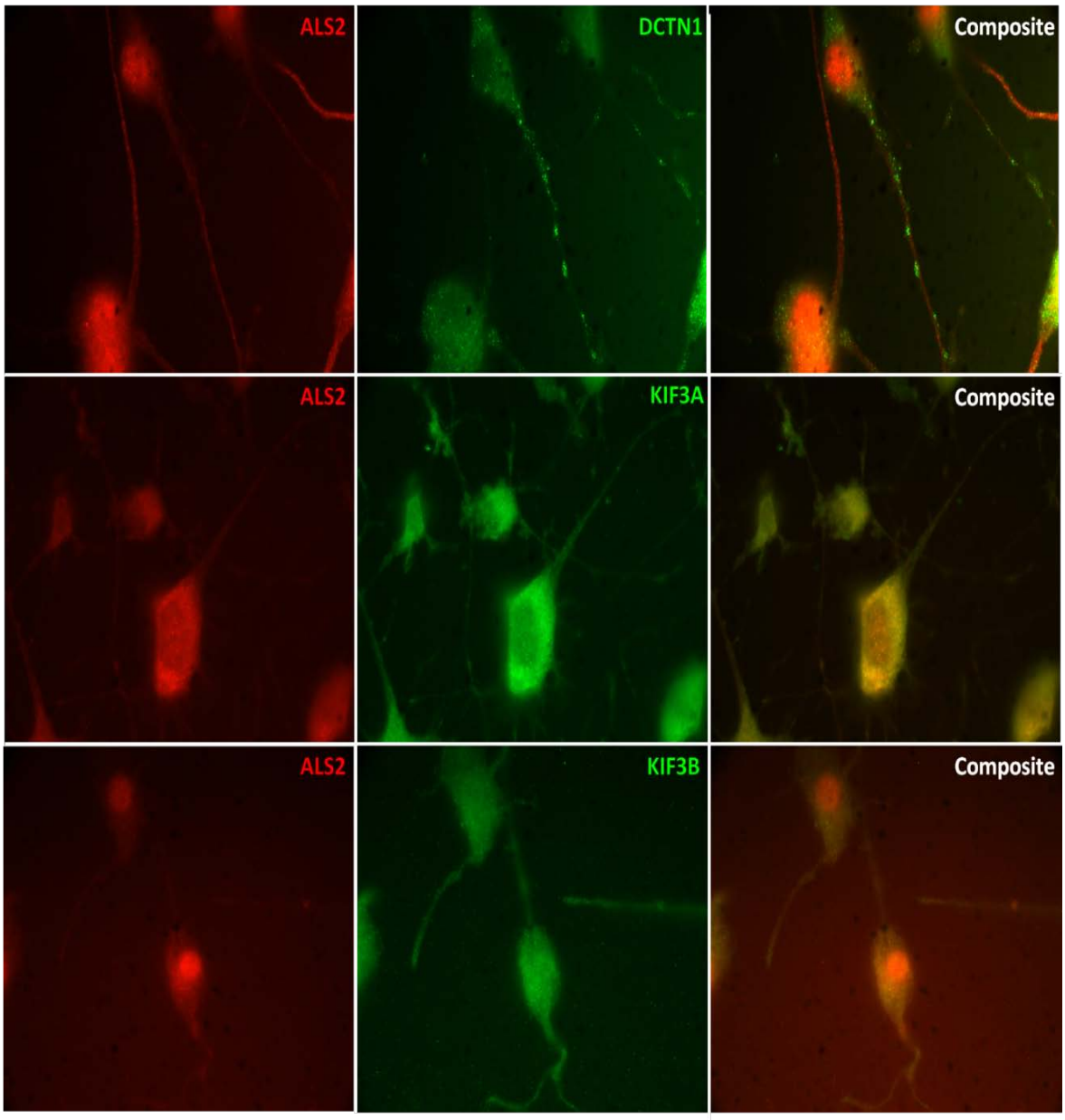

Figure 5.16. Immunofluorescence results displaying the localizations of alsin, DCTN1, KIF3A and KIF3B.

At that point, the absence of bands was questioned by several trouble shooting approaches. First, the proteins loaded to SDS gels were qualified by Coomassie Brilliant Blue to eliminate any possible errors from the protein isolation step; protein quality was as expected (Figure 5.17.).

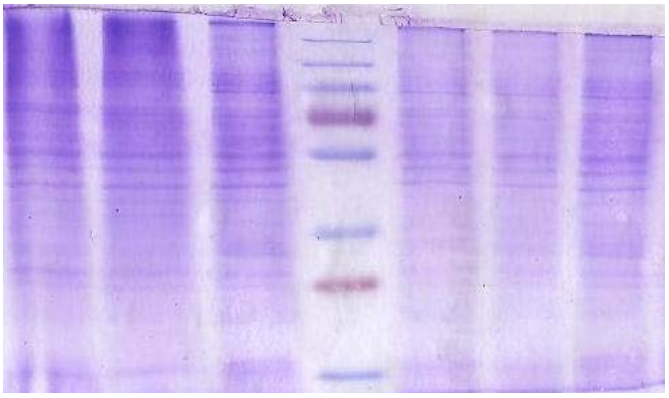

Figure 5.17. Total protein qualification of the samples isolated from alsin-kd and alsin-cnt cells.

Secondly, the same conditions were applied to detect β-actin protein in order to exclude any complications that may have occurred during WB analyses. The bands were detected at 40 kDa as expected (Figure 5.18.).

Figure 5.18. β-actin proteins detected at 40 kDa.

5.2.6 Generation of Alsin-Overexpressing Stable Cell Lines

Considering the probability that low levels of alsin may be the problem, alsin overexpressing stable cell lines (alsin-oe) were established with N2a cells. The overexpression efficiency was measured by q-RT PCR. The results were normalized with β-actin and compared with wt-N2a control cells (alsin-cnt): An 816 per cent increase in expression of alsin was observed in alsin overexpressing cells (Figure 5.19.).

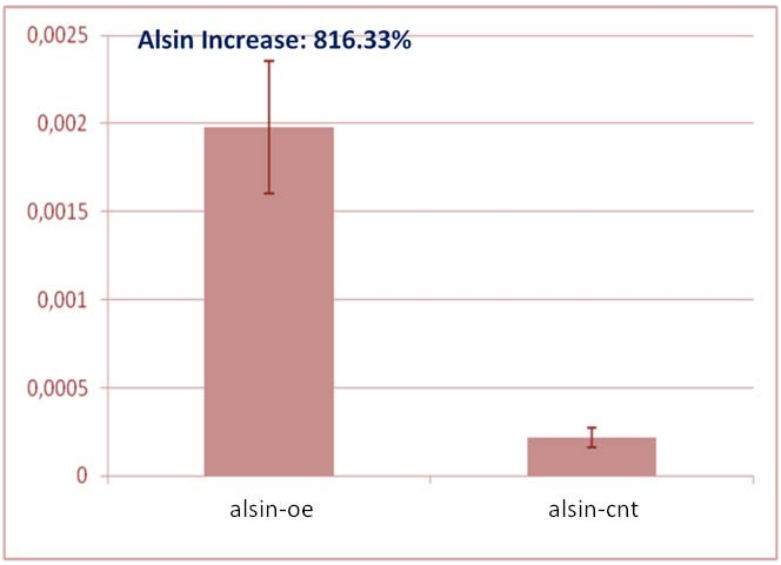

Figure 5.19. Alsin overexpression level of the stable cell lines.

The increase in alsin expression in N2a cells is expected to lead to detectable amounts of alsin protein in future experiments.

6. DISCUSSION

The alsin gene has been associated with several juvenile onset recessive motor neuron diseases, including juvenile Amyotrophic Lateral Sclerosis, Juvenile Primary Lateral Sclerosis and Infantile-onset Ascending Hereditary Spastic Paraplegia (Hadano *et. al.*, 2001; Yang *et. al.*, 2001; Devon *et. al.*, 2003). The homozygous presence of the mutations in all affected patients and the presence of heterozygous unaffected siblings suggest a loss of function paradigm for the alsin protein (Gros-Louis *et. al.*, 2003).

Alsin is a ubiquitously expressed 184 kDa protein, abundant in CNS, especially in cerebellum. It has two different transcripts due to its alternative splicing. Most of the known mutations in the alsin gene are deleterious mutations that lead to the premature termination of the protein (Gros-Louis *et. al.*, 2003).

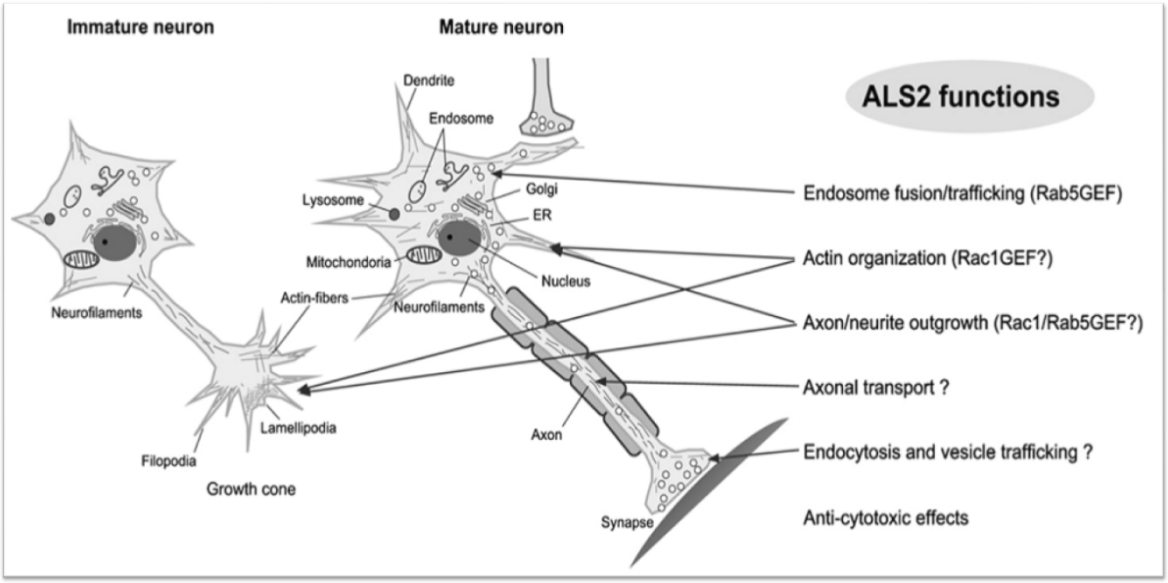

Figure 6.1. Proposed functions of alsin in developing and matured neurons (Hadano *et. al.*, 2007).

The function of alsin is assumed to be regulating/activating multiple small GTPases, as it contains putative GEF domains: RLD, DH/PH and VPS9. Since, almost all mutations lead to the truncation of the VPS domain, alsin's most important function is suggested to

be the Rab5 GEF activity. Additive function hypothesis arose from further studies of alsin as described in Section 1.3.2. (Figure 6.1.).

Despite the loss of function mechanism proposed for alsin-associated motor neuron disorders, alsin knock-out transgenic mice models failed to represent any disease-specific phenotype (Cai *et. al.,* 2005). This evidence points to the importance of investigating the functions and the possible interactions of the alsin protein with other proteins. In the framework of this thesis, the effects of mt-alsin on MNs and the possible interactions of alsin with motor proteins was investigated.

6.1. Effects of Mutant Alsin on Motor Neurons

In this part of the study, three experiment groups (NSC34 cells transfected with the empty vector, wt-alsin and mt-alsin) were set up in order to observe any phenotypical abnormalities in the mt-alsin-transfected group. Although the alsin mutation in this study was generated artificially, it led to an early stop codon formation with the truncation of one third of the protein, the VPS domain, as it is the case in the majority of the identified mutations (Lai *et. al.*, 2009).

The NSC34 cells, transfected and incubated for 24 hours with the control vector, showed the highest transfection efficiency (twice as high as the wt-alsin, and five times higher than the mt-alsin) with a brighter and denser GFP fluorescence diffused in the cytoplasm. Alteration in the transfection efficiencies of the experiment groups could be due to the sizes and/or expression efficiencies of the GFP, wt- and mt-alsins inserted vectors. GFP is a small protein compared to GFP-tagged wt- and mt-alsins. It is reasonable that the intake of the control plasmid to the cells, thus, the number of cnt cells with the GFP signal is higher than the wt- or the mt-alsin-transfected cells. Also, the higher expression efficiency of the small GFP gene compared to the GFP-tagged alsins may be interpreted as a higher transfection efficiency. The faint GFP fluorescence of the wt- and mt-alsin-transfected cells may be due to the masked GFP protein by the alsin protein. While these assumptions could point to highest transfection efficiency in the cnt group, further experiments were required to explain the apparent difference between wt- and mt-alsins' efficiencies.

The transfection efficiencies of all three groups (EG1, 2, 3) were found to be decreased after 48 hours, however, the decrease in mt-alsin was more drastic. The general decrease is not unexpected, since the foreign proteins inserted in the cells are likely to be degraded. It is proposed in several studies that both the alternatively spliced form of alsin and the truncated mutant alsin have very short half-lives and they are difficult to detect in patient samples (Chandran *et. al.*, 2007; Yamanaka *et. al.*, 2003). Like the mt-alsin, wt-alsin used in this study, might also be targeted for a rapid degradation, because of its extra GFP tag.

Effects of mt-alsin on the motor neurons were examined by comparing three phenotypic features in the mt-alsin group with the cnt and wt-alsin groups. Cell viability and defects in axons were selected, considering the suspected involvement of alsin in endosomal and axonal trafficking (Chandran *et. al.*, 2007). In addition to these two features, mitochondrial disorganization was examined, since alsin overexpression was shown to disrupt the mitochondrial trafficking (Millecamps *et. al.*, 2005).

Our results showed no significant phenotypic differences in the selected features of mt-alsin, except the aggregate-like staining of the protein. While the wt-alsin showed a faint but diffused green signal and was localized mostly to the perinuclear region of the cells, mt-alsin presented as bright dots accumulated in the cytoplasm. These results suggest the aggregation of the mt-alsin; this in term explains the difference in the transfection efficiency between the wt- and mt-alsins, and the drastic decline in the mt-alsin amounts after 48 hours transfection.

To confirm the above evidence, Western Blot analysis was performed. A GFP antibody was used to detect the GFP and GFP-tagged alsins. An actin antibody was used as internal control for the protein levels, since actin is a housekeeping gene whose expression is assumed to stay constant under different circumstances. Supporting the results from the fluorescence microscopy, the smear-like image at 24 hours and the absence of mt-alsin at 48 hours, indicated a rapid degradation of the protein, following its aggregation in the cytoplasm.

Protease inhibition to stop degradation of the mt-alsin was performed in the transfected cells as a further confirmation step. Since the inserted protein levels were higher at 24 hours than at 48 hours, 24 hour incubation was preferred. In the protease-inhibited transfected cells, as opposed to the previous WB result, also mt-alsin was detected (below the 200 kDa marker band). In contrast in protease-uninhibited transfected cells, neither wt- nor mt-alsins were detected and the GFP amount was decreased. With fluorescence microscopy, no significant changes were detected between the protease-inhibited and uninhibited cnt groups. However, in wt- and mt-alsins, more green cells were observed, and the green signal intensity was increased in the protease-inhibited cells.

In this part of the study, a rapid degradation of the mt-alsin through the protease pathway was emphasized. The degradation of the wt-alsin was unexpected, but this could be explained by the extra GFP, tagged to the protein. Thus, in future, it would be worth to repeat the experiments with the untagged wt- and mt-alsins, in order to eliminate the probability of a GFP-dependent degradation.

As a consequence of the short half-life and the rapid degradation of the GFP-tagged wt- and mt-alsins, it was very difficult to repeat the results. The smear image of the mt-alsin was detected only twice after several repetitions of the experiment. However, since our fluorescence results and several previous studies in the literature have reported the rapid degradation of mt-alsin, the results presented here are in accordance (Hadano *et. al.*,2007; Chandran *et. al.*, 2007).

6.2. Possible Interactions of Alsin with Motor Proteins

Alsin's RLD domain has been identified in many other proteins with diverse functions, proposing RLD to be a protein-interacting domain (Hadano *et. al.*, 2007). Two missense mutations in this domain were identified in motor neuron diseases, suggesting that the protein interactions of alsin have a very important role in its proper function (Chandran *et. al.*, 2007). Thus, it is crucial to find out alsin's interactors in order to unravel its exact function. Additionally, the absence of disease phenotype in alsin knock-out transgenic mice supports the need to investigate further functions and possible interactors

of alsin. In this respect, the second part of the study was designed to analyze the possible interactions of alsin with several motor proteins.

The reason why the study focuses on the motor proteins is the selective vulnerability of motor neurons in ALS and related disorders. Alsin, like many other MND-associated genes, is expressed ubiquitously in CNS and non-neuronal tissue; however, the mutations cause only motor neuron degeneration (Chandran *et. al.*, 2007). The cell size and the long axons are the two outstanding features of these cells, pointing to the possibility of the selective degeneration due to defects in axonal transport (Morfini *et. al.*, 2009).

The first step of this part included the examination of the expression levels of the motor proteins in the absence of alsin, in alsin-kd stable N2a cell line. The transfected cells gained resistance to hygromycin while the untransfected cells died when exposed to the above antibiotic. These cells were differentiated by serum deprivation and total RNAs were isolated; then they were analysed for the expression levels of the DCTN1, DCTN2, DCTN3, KIF3A, KIF3B and KIF5A genes by q-RT PCR. All q-RT PCR analyses were repeated three times and compared with the expression levels in the control cells. Figures 5.8.- 5.14. and 5.18. in Section 5.7. were derived from the averages of the consistent replicas.

The reason for starting with the expression levels of the six proteins was to observe any changes in the protein levels of the selected genes in the absence of alsin. Although mRNA expression does not always account for an alteration in the protein level, it is a good starting point as it represents the first step of the protein synthesis. In the second step, direct interactions of the alsin protein with DCTN1, KIF3A and KIF3B, showing significant changes in their expression levels, were investigated.

First, localizations of the proteins were examined by IF analyses. All six proteins generally showed a diffused staining in the cytoplasm as expected. Alsin showed a denser localization in the perinuclear region as previously reported in publications (Hadano *et. al.*, 2007; Yamanaka *et. al.*, 2003). The composite images of DCTN1 and KIF3B displayed separate localization, whereas KIF3A showed an obvious co-localization with alsin in the perinuclear region. As mentioned before, KIF3A is the protein binding subunit of the

kinesins, thus it is likely that it interacts with the RLD domain of alsin. Further experiments should be performed in order to confirm this finding.

Next, IP studies were performed in order to detect a direct interaction between alsin and the selected motor proteins. This approach was not successful in presenting specific bands in the WB analyses. At that point, the absence of bands was questioned by several troubleshooting approaches. First, the proteins loaded to SDS gels were qualified by the Coomassie Brilliant Blue, to eliminate any possible errors from the protein isolation step. The results did not point to any abnormalities, deriving from the protein isolation or the loading steps. Secondly, complications that may have occurred during WB analyses were considered, but the β-actin protein detected by the same conditions excluded this probability.

In the light of the above evidences, the problem could be explained by two possibilities: The antibodies used might be dysfunctional, or the amount of alsin protein in N2a cells might be very low such that, despite the presence of the protein, WB analysis fails to detect it. It was previously proposed that it is very difficult to detect endogenous alsin through immunocytochemistry techniques due to the lack of alsin-specific antibodies on one hand (Chandran *et. al.*, 2007) and due to the fact that the endogenous alsin protein is rare even in neuronal tissues (representing 0.0003 per cent of the total protein of the mouse brain) (Yamanaka *et. al.*, 2003).

To overcome the problem of low alsin level in the cells, alsin overexpressing stable cell lines (alsin-oe) were established with N2a cells. Since this part of the study aims to demonstrate the direct interactions between alsin and the DCTN1, KIF3A and KIF3B proteins *in vitro*, the defects caused by the alsin overexpression explained in Section 1.3.2. are negligible here.

Possible interactions between alsin and the DCTN1, KIF3A and KIF3B were implied according to the changes in mRNA levels. As mentioned before, a change in the mRNA level does not always result in an alteration in the protein level. Thus, as a future aspect of the study, WB analysis should be performed to compare the protein levels expressed from the selected genes in the alsin-kd with the control-N2a cells. Although this approach was

initiated in the framework of this study, no result was obtained due to antibody dysfunction. The selection of new antibodies is expected to overcome this problem in future studies. Considering the possibility of a low alsin level in cells, the interaction between alsin and the selected motor proteins should be questioned by repeating the immunoprecipitation approach in alsin-oe cell lines, as a further future experiment.

6.3. Conclusion

The aim of this study was to investigate the functions of alsin, a protein involved in different motor neuron disorders. The experiments performed in this thesis suggest a rapid degradation of the mutant alsin. The results disagree with the previous publications that propose the specific importance of the VPS domain with the Rab5 GEF activity in disease pathogenesis (Lai *et. al.*, 2009). The rapid degradation of the mt-alsin suggests that the pathogenesis of the disease might be due to the overall loss of alsin protein and not only to the loss of the VPS domain. For instance, the RLD is known to be the protein-interaction domain of alsin, and there are two missense mutations described in the RLD domain leading to MNDs, thus making this domain a good candidate for further investigations.

This study also implicates a possible interaction of alsin with DCTN1, KIF3A and KIF3B due to the changes in their expression levels in the absence of alsin. Although the efforts to show a direct interaction between these proteins were not yet reached in the framework of this thesis, immunolocalization results showed a co-localization of KIF3A and alsin in the perinuclear region of N2a cells. This finding is very interesting as the KIF3A is the protein interacting subunit of the kinesins. KIF3A and RLD domain-focused studies could be promising as future aspects.

The results implicating possible interactions between alsin and selected motor proteins should be further elaborated by using alsin-oe cells. In addition, truncated versions of alsin could be generated in order to investigate which domain/domains of the protein are relevant to disease pathogenesis.

This thesis is a first attempt to elucidate the alsin-interacting partners in cell culture systems; we hope that it will help to pave the ways for understanding the complicated and multilayered functions of the alsin protein.

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