Protein Network Analysis Reveals a Functional Connectivity of Dysregulated Processes in ALS and SMA

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ABSTRACT: Spinal Muscular Atrophy (SMA) and Amyotrophic Lateral Sclerosis (ALS) are neurodegenerative diseases which are characterized by the loss of motoneurons within the central nervous system. SMA is a monogenic disease caused by reduced levels of the Survival of motoneuron protein, whereas ALS is a multi-genic disease with over 50 identified disease-causing genes and involvement of environmental risk factors. Although these diseases have different causes, they partially share identical phenotypes and pathomechanisms. To analyze and identify functional connections and to get a global overview of altered pathways in both diseases, protein network analyses are commonly used. Here, we used an \textit{in silico} tool to test for functional associations between proteins that are involved in actin cytoskeleton dynamics, fatty acid metabolism, skeletal muscle metabolism, stress granule dynamics as well as SMA or ALS risk factors, respectively. In network biology, interactions are represented by edges which connect proteins (nodes). Our approach showed that only a few edges are necessary to present a complex protein network of different biological processes. Moreover, Superoxide dismutase 1, which is mutated in ALS, and the actin-binding protein profilin1 play a central role in the connectivity of the aforementioned pathways. Our network indicates functional links between altered processes that are described in either ALS or SMA. These links may not have been considered in the past but represent putative targets to restore altered processes and reveal overlapping pathomechanisms in both diseases.

KEYWORDS: Neurodegenerative disease, fatty acid metabolism, B-Raf, KLF15, actin dynamics, stress granules

Introduction

Degeneration of motoneurons is central in both neurodegenerative diseases Amyotrophic Lateral Sclerosis (ALS) and Spinal Muscular Atrophy (SMA). The underlying mechanisms which result in loss of motoneurons are not fully understood. However, previous studies suggest that identical cellular processes and pathways are altered in both diseases and may contribute to motoneuron degeneration.\textsuperscript{1} In ALS and SMA, alterations in actin dynamics, inflammatory responses as well as altered signaling pathways have been described as such central molecular mechanisms (see reviews\textsuperscript{3-5}).\textsuperscript{1-5}

ALS is characterized by the degeneration of upper and lower motoneurons within the central nervous system (CNS).\textsuperscript{2} Patients suffer from different symptoms including atrophy in proximal and distal muscles, spasticity and cognitive deficits.\textsuperscript{2} In most cases, the prognosis is poor and patients die within 3 to 5 years after diagnosis due to respiratory failure.\textsuperscript{2} Today, no cure for ALS is available but few drugs are approved which enhance patient’s life quality.\textsuperscript{2} ALS is divided into a sporadic (sALS) and a familiar (fALS) form, while the latter one comprises only 10\% of all ALS cases.\textsuperscript{2} Mutations in over 50 different genes and involvement of environmental factors make ALS a multifactorial disease.\textsuperscript{6} Most frequent are gene mutations in \textit{Chromosome 9 open-reading frame 72} (\textit{C9orf72}), \textit{Superoxide dismutase 1} (\textit{SOD1}), \textit{Fused in sarcoma} (\textit{FUS}) and \textit{TAR DNA-binding protein 43} (\textit{TDP–43}). The translated proteins often form protein aggregates which are characteristic for ALS.\textsuperscript{2} Symptoms as well as clinical progression and manifestation are often heterogeneous, making ALS a very complex disease. ALS patients do not only show motoneuron-associated phenotypes, but also alterations in non-neuronal cells.\textsuperscript{7}

SMA was initially described as a neurodegenerative disease with neuronal phenotypes only.\textsuperscript{8} In the last years this assumption has changed since studies showed pathological changes in peripheral organs, for example, skeletal muscle,\textsuperscript{9} liver,\textsuperscript{10} heart,\textsuperscript{11} bone,\textsuperscript{12} pancreas,\textsuperscript{9} and kidney.\textsuperscript{12} Thus, SMA is now considered to be a multi-organ disease (see reviews\textsuperscript{8,13,14}).\textsuperscript{8,13,14} SMA mainly affects children who show degeneration of the second motoneurons within the ventral horn of the spinal cord and brain stem resulting in atrophy of distal muscles.\textsuperscript{15} On the genetic level, cause of the disease are point mutations or deletion of the \textit{Survival of Motoneuron 1} (\textit{SMN1}) gene.\textsuperscript{16} Humans possess a second \textit{SMN} gene, termed \textit{Survival of Motoneuron 2} (\textit{SMN2}). In contrast to \textit{SMN1}, \textit{SMN2} exhibits a cytosine to thymine transition within exon 7.\textsuperscript{17} Due to the point mutation, exon 7 is skipped and a truncated, not fully functional \textit{SMN}\text{Δ}7 protein is translated which is instable and degraded.\textsuperscript{18} Only 10\% of translated \textit{SMN} protein of the \textit{SMN2} gene is fully functional, therefore, \textit{SMN2} only partially compensates the \textit{SMN1} loss.\textsuperscript{8,17,18} In SMA, there is a correlation between...
severity of disease and the number of SMN2 gene copies—a higher copy number leads to a milder phenotype. SMA is divided into 4 clinical subtypes classified by disease onset and achieved motor milestones. In addition, there is a very severe type 0 which is often prenatally lethal.

Here, we employed an in silico protein network analysis of different pathways which have been associated with ALS and/or SMA. The selected pathways included fatty acid metabolism in SMA, B-Raf signaling in different SMA models, the Krüppel-like factor 15 transcription factor that is involved in muscle metabolism, ALS-associated proteins, actin cytoskeleton-related or stress granule dynamics-related proteins, respectively. This global approach aims to identify connections between both neurodegenerative diseases and to reveal potential connections which have not been considered in previous studies. The network approach showed that only a few edges are necessary to build a network that includes various relevant disease factors. Moreover, we identified Superoxide dismutase 1 and the actin-binding protein profilin1 as central nodes which connect diverse pathways.

Material and Methods
Multiple protein network analysis was performed with STRING, an open-access online tool (https://string-db.org; version 11.0). We performed a structured PubMed search with the below mentioned terms with a final evaluation date on June 3rd, 2021. Input protein selection was performed as followed.

The most common genes which are associated with ALS (C9orf72, FUS, TDP-43 and SOD1) and SMA (SMN1 and SMN2) were selected. Besides FUS and TDP-43, SMN is also a DNA/RNA-binding protein, which may indicate a common pathomechanism in both neurodegenerative diseases. As ALS is a multi-genic disease, additional RNA-binding proteins which have been shown to be ALS-associated were added to the list namely TAF15, EWSR1, and MATR3. Interestingly, interactome analysis revealed an interaction of the U1 small nuclear ribonucleoprotein particle (U1 snRNP) with FUS, EWSR1, TAF15, and MATR3. Notably, one of SMN’s housekeeping function is the assembly of snRNPs. U1 snRNP and RNA Polymerase II associate with the Activating Signal Cointegrator 1 (ASC-1) complex. However, ALS- or SMA-causative mutations result in impaired fusion. Another protein that is involved in RNA processing and mutated in ALS is Senataxin (SETX). Decreased expression of SETX has been described in an SMA cell culture model and patient cells. Moreover, this protein may link SMA and ALS as it has been shown that SETX together with zinc finger protein 1 is involved in resolution of co-translational RNA-DNA hybrids, also known as R-loops, and that this process is altered in SMA and ALS models.

For cytoskeletal proteins “actin cytoskeleton” AND “ALS” AND “SMA” AND “motoneuron” terms were used for PubMed search. Profilin is mentioned to be a central regulator in actin dynamics. In SMA, profilin is phosphorylated by ROCK, while under physiological conditions this kinase targets cofilin. Therefore, ubiquitously expressed profilin1, neuronal profilin2, muscular cofilin2, and actin alpha 1, which is the skeletal isoform of actin, were added to the list for the protein network analysis.

SMN is expressed ubiquitously and SMA displays symptoms in multiple organs. Therefore, a publication which address this topic was reviewed. As described in “Spinal muscular atrophy: a motor neuron disorder or a multi-organ disease” by Shababi et al. (2014), SMA patients show metabolic defects, while abnormal fatty acid metabolism is most frequent in SMA type I patients. Hence, a PubMed search with the terms “fatty acid metabolism” AND “SMA” was performed and identified a study about liver data from SMA patients and SMA mouse models. For the network approach, we chose those genes which were identified to be more than three-fold up- or down-regulated, respectively, in liver of intermediate (Smnnos2/2) and severe (Taiwanese) SMA mouse models by a microarray quantitative real-time polymerase chain reaction (qRT PCR) assay (Table 1, column “fatty acid metabolism”).

Restoration of altered pathways in peripheral tissues may be a potential option for further SMA treatment strategies. Therefore, we searched for publications which address this topic. As many skeletal muscles become atrophic in SMA, a PubMed search with the following terms: “spinal muscular atrophy” AND “peripheral tissue” AND “pathway” AND “muscle” AND “therapy.” Six different papers matched with the keywords. One focuses on spinal and bulbar muscular atrophy and two address SMA. Both SMA papers focus on the transcription factor Krüppel-like Factor 15 (KLF15). Therefore, KLF15 and its interaction partners Signal transducer and activator of transcription 3 (STAT3) or Phosphoinositide-3-kinase regulatory subunit 3 (PIK3R3), respectively, were added to the list for STRING analysis (Table 1). KLF15 interactors were identified by STRING Interaction Network section on GeneCards website (https://www.genecards.org/; evaluation date on June 3rd, 2021). Seven STAT family members are expressed in mammals, including two STAT5 isoforms, termed STAT5A and STAT5B. The former isoform is phosphorylated by extracellular signal-regulated kinase (ERK), which is upregulated in SMA. Therefore, STAT5A was included in the network analysis.

Previous studies described that stress granule dynamics is related to ALS and SMA. Therefore, HuR, which is a marker for stress granules, and Ataxin 2, which is associated with stress granules as well as ALS, were selected for the network analysis.

Motoneuron degeneration is central for SMA and ALS and a rescue of altered pathways within these cells can be a potential treatment strategy. The current approved drugs for SMA focus on SMN restoration or splicing modification only. During disease progression such strategies are unable to rescue processes which have become SMN-irreversible. Therefore, combination of direct SMN restoration and
Table 1. Protein list for functional network analysis with STRING. SMN is represented in the network by the genes SMN1 and SMN2. "Other functions" column represents binding partners of KLF15 (#) or components of the U1 snRNP (§).

| Alternative Protein Name | ALS | SMA | Actin Cytoskeleton | Fatty Acid Metabolism | SG Dynamics | Other Functions |
|--------------------------|-----|-----|--------------------|-----------------------|-------------|-----------------|
| 14-3-3ζ/δ               |     |     |                    |                       |             |                 |
| YWHAZ; Tyrosine 3-monooxygenase/Tryptophan 5-monooxygenase activation protein zeta | x   |     |                    |                       |             |                 |
| ACTA1                    |     |     |                    |                       |             |                 |
| Actin alpha 1            |     |     |                    |                       |             | x               |
| ATXN2                    |     |     |                    |                       | x           |                 |
|                         |     | x   |                    |                       |             |                 |
| B-Raf                    |     |     |                    |                       |             |                 |
| B-Raf proto-oncogene; Serine/threonine kinase | x   |     |                    |                       |             |                 |
| C9orf72                  |     |     |                    |                       |             |                 |
| Chromosome 9 open-reading frame 72 | x   |     |                    |                       |             |                 |
| CD36                     |     |     |                    |                       |             |                 |
| Fatty acid translocase; Platelet Glycoprotein IV | x   | x   |                    |                       |             |                 |
| CFL2                     |     |     |                    |                       |             |                 |
| Cofilin2                 |     |     |                    |                       |             | x               |
| EWSR1                    |     |     |                    |                       |             | §               |
| EWS RNA-binding protein 1|     |     |                    |                       |             | §               |
| FABP1                    |     |     |                    |                       |             | x               |
| Fatty acid binding protein 1 (liver) |     |     |                    |                       |             | x               |
| FABP3                    |     |     |                    |                       |             | x               |
| Fatty acid binding protein 3 (heart) |     |     |                    |                       |             | x               |
| FABP5                    |     |     |                    |                       |             | x               |
| Fatty acid binding protein 5 (epidermal) |     |     |                    |                       |             | x               |
| FUS                      |     |     |                    |                       |             | §               |
| Fused in sarcoma; RNA-binding protein | x   |     |                    |                       |             | §               |
| G6PC                     |     |     |                    |                       |             |                 |
| Glucose-6-phosphatase    |     |     |                    |                       |             | x               |
| HuR                      |     |     |                    |                       |             | x               |
| ELAVL1; Human antigen R  |     |     |                    |                       |             |                 |
| IFNG                     |     |     |                    |                       |             | x               |
| Interferon gamma         |     |     |                    |                       |             |                 |
| IGF1                     |     |     |                    |                       |             | x               |
| Insulin-like growth factor 1 |     |     |                    |                       |             | x               |
| IGF1P                    |     |     |                    |                       |             | x               |
| Insulin-like growth factor-binding protein 1 |     |     |                    |                       |             | x               |
| KLF15                    |     |     |                    |                       |             |                 |
| Krüppel-like factor 15   |     |     |                    |                       |             |                 |
| LDLR                     |     |     |                    |                       |             | x               |
| Low-density lipoprotein receptor |     |     |                    |                       |             |                 |
| LPL                      |     |     |                    |                       |             | x               |

(Continued)
### Table 1. (Continued)

| (ALTERNATIVE) PROTEIN NAME | ALS | SMA | ACT IN CYTOSKELETON | FATTY ACID METABOLISM | SG DYNAMICS | OTHER FUNCTIONS |
|----------------------------|-----|-----|---------------------|-----------------------|-------------|-----------------|
| MATR3 Matrin-3             |     | x   |                     |                       |             | §               |
| PDK4 Pyruvate dehydrogenase kinase 4 |     |     |                      | x                     |             |                 |
| PFN1 Profilin1             |     |     |                     |                       |             |                 |
| PFN2 Profilin2             |     |     | x                   | x                     |             |                 |
| PIK3R3 Phosphoinositide-3-kinase regulatory subunit 3 |     |     |                      |                       |             | #               |
| PKLR Pyruvate kinase L/R   |     |     |                     |                       |             |                 |
| SETX Senataxin             |     |     |                     |                       |             |                 |
| SLC27A5 Bile acyl-CoA synthetase |     |     |                     |                       |             |                 |
| SMN1 Survival of Motoneuron 1 |     |     | x                   |                       |             |                 |
| SMN2 Survival of Motoneuron 2 |     |     |                     |                       |             |                 |
| SOD1 Superoxide dismutase 1 |     |     |                     |                       |             |                 |
| SREBF1 Sterol regulatory element-binding transcription factor 1 |     |     |                     |                       |             |                 |
| STAT3 Signal transducer and activator of transcription 3 |     |     |                     |                       |             | #               |
| STAT5A Signal transducer and activator of transcription 5 A |     |     |                     |                       |             |                 |
| TAF15 TATA-box binding protein associated factor 15 |     |     | x                   |                       |             | §               |
| TDP-43 TAR DNA-binding protein 43 |     |     |                     |                       |             | x               |
SMN-independent pathways could be a more suitable approach for treatment in later stages of the disease. Thus, we searched for studies which matched with the following terms: “motoneuron degeneration” AND “rescue” AND “SMN-independent.” Two results were identified in PubMed search, including one paper with an unbiased approach focusing on alterations and rescue approaches in several SMA models. In SMA, neurotrophic signaling is dysregulated in a network, in which the serine/threonine kinase B-Raf is central. Next to B-Raf, its binding partner 14-3-3 δ/ζ, which was also shown to be downregulated in SMA, was added to the protein network list.

A summary of the selected proteins is listed in Table 1. Proteins were submitted to STRING software and *Homo sapiens* was chosen as reference organism. Text-mining, Experiments, Databases, Co-expression, Neighborhood, Gene Fusion, and Co-occurrence were selected as channels. The medium required interaction score was set to medium confidence (0.4). Moreover, maximal number of interactors of second shell was set to “no more than 5 interactors.” Selection of second shell parameter resulted in inclusion of additional proteins (Table 2).

**Results**

SOD1 and PFN1 are inter-modular nodes in a protein network which comprises proteins related to ALS, SMA, fatty acid metabolism, B-Raf signaling, and stress granule as well as actin dynamics.

Network biology is a powerful tool for presentation of functional interactions of proteins. In the last years, this *in silico* approach has been often used to understand complex biological interactions of proteins. Moreover, network biology supports researchers to identify potential new treatment strategies for neurodegenerative diseases. Here, we performed a network analysis on published studies that focus on fatty acid metabolism in SMA patients and mouse models. KNF15 overexpression in an SMA mouse model and B-Raf signaling in SMA. Moreover, further proteins were added that are involved in actin and stress granule dynamics as well as the most common ALS-associated proteins (Table 1). Network analysis with STRING revealed that some proteins form clusters (Figure 1). These clusters are related to fatty acid metabolism, actin cytoskeletal proteins (Figure 1, orange circle) or ALS-associated proteins (Figure 1, blue circle), respectively. According to Gene Ontology (GO) analysis, 251 GO-terms of biological processes, 9 GO-terms of molecular function and 15 GO-terms of cellular component were significantly enriched (Table 3; for clarity only the top 10 hits for biological processes, 9 GO-terms of molecular function and 15 GO-terms of cellular component were significantly enriched). Moreover, SOD1 and PFN1 were identified as inter-modular nodes which connect different clusters. Interestingly, SOD1 is not only linked to fatty acid metabolism via FABP3, FABP5, and IGF1 but also to the cluster of actin cytoskeleton proteins, ALS-associated proteins as well as $\text{SMN1}$ and $\text{SMN2}$. A multi-connectivity of proteins (PFN1 and SOD1) revealed that those proteins do often not only have functions in one specific pathway/process but are simultaneously linked to various biological processes.

**Discussion**

Processes explaining the mechanisms behind motoneuron degeneration in ALS and SMA are not fully understood. However, several mechanisms have been described and factors either associated with or being modified of the diseases have been identified. While SMA is a monogenic disease, ALS is caused by different factors. Furthermore, some proteins or members of protein families have been associated with both diseases for example cytoskeletal or stress granule-associated proteins. Interestingly, SMN protein level is also associated with ALS, as shown by ALS patient studies. However, functional protein network analysis illustrates mechanisms that are linked to ALS as well as SMA. Here, we employed a protein network analysis of proteins which are involved in fatty acid as well as muscle energy metabolism, B-Raf signaling, actin cytoskeleton organization, stress granule dynamics, and SMA- or ALS-associated proteins, respectively. Our data showed that only a small number of edges, which display connections between single proteins represented as nodes, are necessary to connect various molecular processes. Moreover, we identified SOD1 and PFN1 as central inter-modular nodes (hubs) within this network.

One cluster of the network is represented by proteins involved in actin dynamics (Figure 1, orange circle). Actin dynamics is a tightly regulated process that facilitates cellular survival and proper function. Alterations within this system trigger motoneuron degeneration in SMA and ALS. The actin-binding protein profilin facilitates actin polymerization. Humans harbor different profilin isoforms including ubiquitously expressed profilin1 and the major neuronal isoform profilin2a. Both isoforms bind the SMN protein. In SMA mice, an altered phosphorylation pattern of actin-binding proteins profilin2a and cofilin have been described with impact on growth cone regulation and neurite formation. Furthermore, single phosphorylations at specific sites in profilins have consequences on the interaction with its binding partners actin, poly-L-proline, or phosphatidylinositol (4,5)-bisphosphate expressing proteins, respectively. These studies showed that posttranslational modifications have a significant impact on binding affinities and proper profilin functions in actin dynamics. Motoneuron degeneration is not only restricted to alterations within profilin-mediated actin dynamics. We previously showed that an altered signaling network with the serine/threonine kinase B-Raf in its center contributes to motoneuron degeneration in SMA patient cells and different SMA animal models. Here, we showed that this network is associated with a cluster of cytoskeletal proteins via 14-3-3 δ/ζ, a binding partner of B-Raf (Figure 1, orange circle). In a severe SMA mouse model, B-Raf and 14-3-3 δ/ζ expression was both decreased. However, B-Raf rescue in C.
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*elegans* successfully enhanced motoneuron number sustaining B-Raf’s role in proper motoneuron function. 22,58 14-3-3 protein displays seven isoforms. Their protein expression has been investigated in frontal cortices of a rodent model for neurodegeneration59 and *post mortem* tissues from Alzheimer’s disease patients,60 revealing a altered expression pattern of different 14-3-3 isoforms and total protein levels.59,60 In rodents, ζ/δ isoforms were less expressed in comparison to the other isoforms, while in humans the β/α isoform had the lowest expression levels.59,60 14-3-3 and B-Raf form a complex together

Figure 1. Network analysis revealed SOD1 and PFN1 as inter-modular nodes in a network representing proteins that are involved in SMA and ALS pathogenesis. Components of the actin cytoskeleton (orange circle) include actin-binding proteins profilin1 and 2 (PFN1 and PFN2) as well as cofilin2 (CFL2) and actin alpha 1 (ACTA1). This cluster is linked to proteins which are associated with ALS (blue circle). SOD1 is an inter-modular node that connects ALS- or SMA-caused proteins, respectively, with the cluster of proteins that are altered in fatty acid metabolism in SMA mice. Most of the shown proteins have functions in fatty acid metabolism. Family members of the STAT kinases (STAT3 and STAT5A) are connected to KLF15 which regulates energy pathways in muscles. B-Raf and its interactor 14-3-3 ζ/δ are linked to the cluster of actin-binding proteins or to STAT kinases via RAF1, respectively. Complete protein names are listed in Tables 1 and 2.
with Mitogen-activated protein kinase kinase 1 (MAP2K1), which is also known as MEK1. The latter protein is part of the mitogen-activated growth factor (MEK)/extracellular signal-regulated kinase pathway (ERK) signaling cascade, which is involved in proliferation, survival and growth inhibitory signaling. In a Caenorhabditis elegans SMA animal model, this pathway-mediated motoneuron degeneration. These studies hint for a direct link of 14-3-3 protein expression and neurodegeneration. However, it remains elusive to identify the underlying mechanism in further studies. Our network biology approach suggests a possible downstream mechanism via actin or cofilin (Figure 1). Interestingly, cofilin binds wild type Chromosome 9 open reading frame 72 (C9orf72) protein, which is mutated in ALS. However, it is unknown whether mutated C9orf72 still binds to cofilin and which consequence this might have on motoneuron survival in ALS. Nevertheless, this interaction together with our reported association leads to the hypothesis that B-Raf signaling may be also altered in C9orf72-mediated ALS, which has to be proven in future studies. In summary, proper functionality, protein expression and regulation of actin-binding proteins is important for motoneuron survival. Altered expression, phosphorylation or binding by other proteins have significant consequences not only on the actin cytoskeleton dynamics but also on downstream processes such as survival and degeneration of motoneurons.

Our network analysis revealed an additional novel link of 14-3-3 ζ/δ with the transcription factor Krüppel-like factor 15 (KLF15) via Phosphoinositide-3-kinase regulatory subunit 3 (PIK3R3) and Signal transducer and activator of transcription 3 (STAT3) (Figure 1). KLF15 regulates energy metabolism including glucose homeostasis and amino acid catabolism. In skeletal muscles the JAK/STAT axis was activated by intercellular communication and the translation of findings from one disease to another. These studies described here show that altered energy homeostasis could be a common characteristic of neurodegenerative diseases.

KLF15 is linked to proteins of the fatty acid metabolism via Sterol regulatory element–binding transcription factor 1 (SREBF1) (Figure 1), which was downregulated in SMA mice. Additionally, KLF15 is linked to B-RAF/14-3-3 signaling by STAT3 (Figure 1), which is a component of the JAK/STAT pathway. The JAK/STAT cascade is activated upon binding of cytokines (eg, IFNG) and growth factors to receptors which activate JAK and phosphorylate downstream STAT proteins, which then regulate the transcription of several genes. Interestingly, STAT3 signaling is involved in microtubule stabilization in the axonal cytoskeleton of motoneurons. Furthermore, in the neurodegenerative disorder Parkinson’s Disease, the JAK/STAT pathway is dysregulated (reviewed in 73), suggesting a similar effect in SMA and ALS. However, the complexity and association of protein interactions is only partly presented by network approaches. Thus, network approaches give an initial overview how isolated clusters are linked to other clusters. STATs upstream activators JAK are involved in immune responses. In ALS and SMA, activation of immune cells has been described. Additionally, in skeletal muscles the JAK/STAT axis was activated by interleukin (IL)-6 which is released during muscle contraction or after muscle injury. Studies in mice showed that an overexpression of IL-6 causes muscle atrophy. Thus, current studies in mice assess the effect of IL-6/JAK/STAT modulation in the context of Duchenne muscular dystrophy. Therefore, a modulation of the JAK/STAT signaling may be another potential treatment strategy for neurodegenerative diseases.

Our network data show that STAT proteins are additionally linked to proteins of the fatty acid metabolism (Figure 1), which is dysregulated in SMA mouse models. While expressions of Fatty Acid Synthase (FASN) and Acat1-CoA Synthetase Long Chain Family Member 5 (ACSL5) were downregulated in SMA mouse models, both genes were unchanged in post mortem cortex tissue of sALS and C9orf72-ALS patients.
Interestingly, expression profiles of Acetyl-CoA Carboxylase Alpha (ACACA) showed contrary results with downregulation in SMA mice and upregulation in sALS post mortem tissue.\(^{10,75}\) This may be due to the fact that different species and age of mice and patients were investigated. Analyzed SMA mice mimic an intermediate model for SMA while post mortem tissue samples represent the end point of the disease in humans. Thus, other SMA mouse models or time points of organ harvesting may show similar results as seen in ALS patients. Together, these studies argue for a similar pathomechanism in both neurodegenerative diseases. This hypothesis is further supported by our global network analysis which revealed an association between fatty acid metabolism proteins and ALS-associated proteins (Figure 1, blue circle). However, additional studies are needed to verify this hypothesis.

As mentioned above, proteins of the fatty acid metabolism are linked to ALS-causing proteins by SOD1 which is also connected to a cluster of cytoskeletal-associated proteins (Figure 1). Energy metabolism studies in the SOD1\(^{G93A}\) ALS mouse model revealed an enhanced fatty acid oxidation in these animals compared to wild type controls.\(^{76}\) Together with the results of the fatty acid metabolism study in SMA,\(^{10}\) this indicates for a common pathomechanism in SMA and ALS. Furthermore, SMN protein levels is associated with ALS as described earlier.\(^{49,50}\) Here, we also showed such a connection within our network analysis (Figure 1). SMN is a ubiquitously expressed protein that localizes to the nucleus and cytoplasm,\(^{27}\) while studies with SOD1\(^{G93A}\) mice demonstrated an irregular distribution of nuclear SMN ranging from normal to absent SMN protein.\(^{27}\) However, this indicates a pathomechanism in ALS based on SMN-related defects in RNA metabolism, in which SMN is involved in multiple steps.\(^{27}\) Interestingly, an overexpression of SMN in the SOD1\(^{G93A}\) mouse model improved ALS phenotypes including elevated motoneuron number and delayed symptom onset.\(^{78}\) Additionally, our network analysis identified SOD1 as a central hub that further connects fatty acid metabolism, SMN protein, ALS-associated proteins and the cluster of actin dynamics-related proteins. Besides the various housekeeping functions of SMN in cytoskeleton dynamics, autophagy and endocytosis, SMN is essential for ribonucleoprotein formation during splicing.\(^{48}\) This interaction is also represented by the protein network in which SETX is linked to SMN (represented in the network by SMN\(^{1}\) and SMN\(^{2}\) genes) (Figure 1). In addition to SMN, FUS and TDP-43 have binding sites for RNAs, too.\(^{52}\) These RNA-binding proteins can localize to membrane-less structures such as stress granules.\(^{27,42}\) After heat shock, endoplasmic reticulum or oxidative stress, respectively, stress granules assemble, and enable cells to save energy by storing mRNA transcripts within these granules and terminate further processing.\(^{79-81}\)

### Table 3. Functional enrichments in the network (Gene Ontology (GO) analysis). Overview of the most significant enriched biological processes (FDR $< 10^{-5}$) and molecular functions (FDR $< 0.01$).

| GENE ONTOLOGY                        | GO-TERM | FALSE DISCOVERY RATE (FDR) |
|--------------------------------------|---------|----------------------------|
| Biological processes                 |         |                            |
| Cellular response to oxygen-containing compound | GO:1901701 | 1.25·10\(^{-9}\)           |
| Response to endogenous stimulus      | GO:0009719 | 1.37·10\(^{-8}\)           |
| Cellular response to chemical stimulus | GO:70887 | 1.37·10\(^{-8}\)           |
| Response to organonitrogen compound  | GO:10243 | 2.07·10\(^{-8}\)           |
| Response to oxygen-containing compound | GO:1901700 | 2.33·10\(^{-8}\)           |
| Cellular response to organonitrogen compound | GO:71417 | 2.85·10\(^{-8}\)           |
| Response to peptide                  | GO:1901652 | 2.87·10\(^{-8}\)           |
| Cellular response to organic substance | GO:71310 | 3.79·10\(^{-8}\)           |
| Cellular response to peptide         | GO:1901653 | 1.22·10\(^{-7}\)           |
| Positive regulation of biological process | GO:48518 | 1.73·10\(^{-7}\)           |
| Molecular function                   |         |                            |
| Long–chain fatty acid transporter activity | GO:0005324 | 2.46·10\(^{-6}\)           |
| Protein binding                      | GO:0005515 | 1.78·10\(^{-5}\)           |
| Identical protein binding            | GO:42802 | 1.98·10\(^{-5}\)           |
| Binding                              | GO:0005488 | 1.98·10\(^{-5}\)           |
| Lipid transporter activity           | GO:0005319 | 4.42·10\(^{-4}\)           |

Abbreviation: FDR, false discovery rate.
However, the stress granule dynamics including formation and disassembly is altered in ALS and SMA, which can result in protein aggregate formation.\(^{9-15}\) In conclusion, the protein network includes several proteins with RNA-binding domains. Moreover, proteins involved in stress granule dynamics are represented. Thus, these results are consistent with a central involvement of RNAs in different neurodegenerative diseases based on inappropriate RNA binding and processing as well as altered stress granule dynamics.

Taken together we showed that proteins of different biological processes, such as actin or stress granule dynamics, respectively, energy metabolisms, or signaling pathways, for example, B-Raf and JAK/STAT, form a protein network which is built by few edges. Furthermore, the actin-binding protein profilin1 and Superoxide dismutase 1 are central hubs within this network. Since these modules are highly connected, modulation of these proteins may be appropriate for restoration of dysregulated pathways. However, the network also shows that pathways cannot be seen as isolated entities, but as an interplay of different clusters. Therefore, combinatorial treatment strategies are favored since single proteins often play a role in different pathways and are linked to several other proteins. Finally, we showed that a network analysis is a powerful tool to get a global overview of the interplay of different clusters.

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**Author Contributions**

Conception and design of the study: SK and PC. Data acquisition: SK. Data analysis: SK and PC. Writing—original draft and visualization: SK. Writing—Review and Editing: SK and PC. Final approval of the manuscript: SK and PC.

**Significance Statement**

Protein network approaches identify links between altered biological processes in ALS and SMA. SOD1 and PFN1 are inter-modular proteins that are highly connected to different clusters.

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