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Author: Heidi R. Fuller, Thomas H. Gillingwater, Thomas M. Wishart

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Commonality amid diversity: multi-study proteomic identification of conserved disease mechanisms in spinal muscular atrophy.

Heidi R. Fuller<sup>a\*</sup>, Thomas H. Gillingwater<sup>b</sup> and Thomas M. Wishart<sup>c\*</sup>

<sup>a</sup>Wolfson Centre for Inherited Neuromuscular Disease, RJAH Orthopaedic Hospital,
Oswestry, SY10 7AG, UK; Institute for Science and Technology in Medicine, Keele
University, Staffordshire, ST5 5BG, UK;
<sup>b</sup>Centre for Integrative Physiology, University of Edinburgh, UK; Euan MacDonald Centre
for Motor Neurone Disease Research, University of Edinburgh, UK;
<sup>c</sup>Euan MacDonald Centre for Motor Neurone Disease Research, University of Edinburgh, UK
Division of Neurobiology, The Roslin Institute and Royal (Dick) School of Veterinary
Studies, University of Edinburgh, UK.

\* Corresponding authors

Email: <u>h.r.fuller@keele.ac.uk</u> Telephone: +44(0)1691 404693 Fax: +44(0)1691 404065

Email: <u>T.M.Wishart@Roslin.ed.ac.uk</u> Telephone: +44(0) 131 651 9100 Fax: +44 (0)131 651 9105

#### Highlights

- · Proteomics highlights potential conserved mechanisms of pathogenesis in SMA
- GAP43, GAPDH, UBA1 and NCAM are decreased in three separate proteomic studies of SMA
- LMNA, ANXA2 and COL6A3 are increased in three separate proteomic studies of SMA
- · Manipulation of these proteins may modulate the severity of disease in SMA

#### <u>Abstract</u>

The neuromuscular disease spinal muscular atrophy (SMA) is a leading genetic cause of infant mortality, resulting from low levels of full-length survival motor neuron (SMN) protein. Despite having a good understanding of the underlying genetics of SMA, the molecular pathways downstream of SMN that regulate disease pathogenesis remain unclear. The identification of molecular perturbations downstream of SMN is required in order to fully understand the fundamental biological role(s) for SMN in cells and tissues of the body, as well as to develop a range of therapeutic targets for developing novel treatments for SMA. Recent developments in proteomic screening technologies have facilitated proteome-wide investigations of a range of SMA models and tissues, generating novel insights into disease mechanisms by highlighting conserved changes in a range of molecular pathways. Comparative analysis of distinct proteomic datasets reveals conserved changes in pathways converging on GAP43, GAPDH, NCAM, UBA1, LMNA, ANXA2 and COL6A3. Proteomic studies therefore represent a leading tool with which to dissect the molecular mechanisms of disease pathogenesis in SMA, serving to identify potentially attractive targets for the development of novel therapies.

**Keywords** 

Spinal muscular atrophy; SMA; SMN; UBA1; proteomics

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#### Introduction

Spinal Muscular atrophy (SMA) is an autosomal recessive disease, primarily characterised by loss of motor neurons from the anterior horn of spinal cord and atrophy of skeletal musculature. The disease is considered to represent a "continuum of clinical severity" [1], but is broadly subdivided into four sub-types, depending on the developmental milestones that are reached: type 1 (severe), type 2 (intermediate), type 3 (mild) and type 4 (adult-onset) [1]. Type 1 SMA is the leading genetic cause of infantile death in the western world and infants typically die before the age of two due to respiratory failure [2,3].

The cause of SMA for the majority (>95%) of patients is a loss-of-function defect in the *SMN1* gene, resulting in reduced levels of the ubiquitously-expressed survival of motor neuron (SMN) protein [4]. Although most humans possess at least one copy of an additional - and almost identical - *SMN2* gene, protein translated from *SMN2* is much less stable and unable to fully compensate for loss of *SMN1* [4-6]. The severity of the disease is largely dependent upon the number of *SMN2* copies that are present. Thus, patients with the most severe phenotypes tend to have a lower copy number of *SMN2* [7].

At present, there are no disease modifying treatments available for SMA, and palliative support is the best that can be offered to patients. However, significant progress has been made over the last two decades in terms of both basic research and pre-clinical development, leading to the identification of several promising therapeutic approaches entering clinical trials. Almost all of this therapeutic work has focused on identifying compounds aimed at targeting either *SMN2* promoter activation, modulation of splicing, or *SMN1* replacement gene therapy [8]. In contrast, the ability to identify potential non SMN-focused therapeutic

targets has been hampered by a lack of understanding of the core molecular pathways acting downstream from SMN to modulate disease pathogenesis in SMA [9].

SMN is a ubiquitously expressed protein with a role in the assembly of small nuclear ribonucleic proteins (snRNPs) in the cytoplasm and subsequent transport into the nucleus for RNA splicing [4,10]. To do this, SMN functions as a core complex with at least eight other proteins: gemins 2-8 and unrip. In addition to this well-characterised housekeeping role, SMN appears to interact with several other proteins required for the transport and correct localisation of mRNAs in axons [11-13]. Whilst some of these SMN interactions are relatively stable (in the case of the core complex with gemin proteins), there are many other interactions that are expected to be more transient [14]. A search of two protein interaction databases, Biomolecular Interaction Network Database (BIND) and the Molecular INTeraction database (MINT), suggests that in excess of 100 proteins have the potential to interact with SMN. Given the potential size of the "SMN interactome" (Figure 1), and the fact that each of those interacting proteins may also have their own unique "interactome", it seems highly probable that a reduction of SMN should have significant downstream molecular consequences affecting a range of different target proteins and pathways.

Recent advances in proteomic technologies have enabled researchers to take an unbiased, global view of the proteome, and to conduct quantitative comparisons and characterisations of disease-relevant tissues and model systems relevant to a broad range of conditions affecting human health. Such technologies therefore provide un-paralleled opportunities to accurately monitor and quantify downstream molecular consequences of SMN depletion *in vivo* and *in vitro*. It is therefore not surprising that proteomics approaches have been

employed by a variety of research groups investigating mechanisms of disease pathogenesis in SMA.

Despite the wealth of information that proteomic investigations can generate, two main challenges have hampered effective translation of findings from studies using these approaches to investigate mechanisms of disease pathogenesis in SMA. The first challenge is that different proteomics-based studies of SMA have utilised a variety of model systems and tissue sampling techniques. This has the potential to result in unavoidable, model/tissuespecific identified perturbations into proteomic data that are difficult to isolate from core molecular disease pathways. The second challenge is that proteomics studies typically yield extensive lists of proteins exhibiting differential expression profiles based on an incomplete coverage of the entire proteome, with a select few candidates from each study often given special attention for further verification and validation. The selection of such candidate proteins for further study is often subjective and will always result in other potentially important proteins and pathways being overlooked. As a result, there are now a number of large proteomic datasets from several research groups that contain crucial, but likely overlooked, information regarding the core molecular regulators of disease pathogenesis in SMA. Despite these challenges, the availability of such a broad range of SMA proteomic datasets presents the opportunity to generate significant new insights into mechanisms of disease pathogenesis by interrogating raw datasets to identify common molecular changes. The identification of conserved alterations downstream of SMN perturbations, independent of the experimental model and technical approach used, would therefore serve to highlight "core" molecular responses to low levels of SMN. Such "core" responses would likely represent attractive SMN-independent targets for the development of novel therapies for SMA.

#### SMA studies employing proteomic screens: An overview

Nine publications to date have used unbiased proteomic comparisons for the identification of differentially-expressed proteins in SMA (Table 1). These studies are considered unbiased because they did not depend on the use of capture or array technology for enrichment of differentially expressed proteins. Instead, the entire proteome was subject to analysis using a variety of protein separation and mass spectrometry approaches. As expected, there is a clear correlation between the precise methodology used and the number of differentially expressed proteins identified in each study; 2D-gel based studies yielded far fewer proteins than more recently-developed isobaric (e.g. iTRAQ) or label-free technologies, even when identical mass spectrometry instrumentation was used. Despite the relatively small number of studies, both in-vivo and in-vitro studies are represented, incorporating experimental assessment of both mouse models and SMA patient samples (Table 1). The studies include comparisons made of mature cells and tissues [15-21], as well as comparisons of cell types at various stages of cellular differentiation, including undifferentiated and differentiated embryonic stem cells [22], induced pluripotent stem cell-derived motor neurons [16], and Schwann cells [23]. Also noteworthy is that three of the studies [15-17] conducted comparisons using material that was not clinically affected by SMA (i.e. plasma and skin fibroblasts), thus enabling the possibility of identifying systemic consequences of reduced SMN in the absence of overt disease pathology. Taken together, these nine published proteomic studies cover a broad range of the most common SMA models, cells and tissues utilised by the research community. Comparisons between and across experimental datasets from these studies are therefore likely to identify conserved molecular responses to low levels of SMN directly relevant to disease pathogenesis.

### <u>Multi-study proteomic identification of conserved molecular responses to reduced levels</u> of SMN in SMA

Review of the differentially expressed proteins from the studies detailed in **Table 1** identified 29 proteins that showed conserved changes across two or more separate proteomic-based studies of SMA (**Table 2**). Of these 29 proteins, 19 revealed increased expression in response to low levels of SMN and 10 revealed decreased expression levels (**Table 2**). These proteins therefore represent a novel potential "molecular fingerprint" of SMA pathogenesis.

Of the 10 proteins that were decreased in expression in SMA cell and tissue samples, four were found to be consistently decreased across three separate studies (**Table 2**), suggesting that they may represent a conserved response to lowered levels of SMN and as a result warrant significant further experimental attention. One of these proteins, ubiquitin activating enzyme 1 (UBA1), has been strongly linked to neurodegenerative pathways across a range of diseases, including SMA [24]. Of particular relevance too is that mutations in the human *UBE1* gene, that encodes UBA1 protein, are associated with a rare X-linked infantile form of SMA [25,26]. Despite differences in experimental platforms, species and tissue / cell type utilised in the quantitative proteomic studies, the percentage reduction of UBA1 is strikingly comparable across all three studies. Aghamaleky *et al.* [23] detected a 50% decrease of UBA1 in SMA mouse hippocampal synaptosomes; Fuller *et al.* [16] detected a 52% decrease in UBA1 in SMA patient iPSC-derived motor neurons.

Further experimental evidence has verified that UBA1 levels are significantly depleted at both the gene and protein level in iPS-derived motor neurons from three individual SMA patients [16], and at the tissue and cellular level in mouse models of SMA. The reduction in

mouse models of SMA contributes, at least in part, to widespread perturbations in ubiquitin homeostasis [20,23,27]. Importantly, experimental suppression of UBA1, using both genetic and pharmacological approaches, was sufficient to fully phenocopy the SMA motor neuron phenotype in zebrafish [20], and pharmacological suppression of UBA1 in normal Schwann cells phenocopied the differentiation/myelination defects observed in SMA Schwann cells [23]. Thus, the robust finding of depleted levels of UBA1 across multiple proteomic studies of SMA suggests that this protein is likely to be a major contributor to disease pathogenesis in SMA.

Also reduced across three separate comparisons was the traditional "housekeeping" glycolytic enzyme, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (**Table 2**). The reduction seen in SMA mouse Schwann cells (56%; [23]) was slightly greater than that seen across the isoforms of GAPDH that were detected in the SMA mouse hippocampus comparison (approximately 40% reduction on average [18]). It was not possible to compare the average percentage reduction seen in SMA patient plasma samples with the other studies because proteins were expressed according to their correlation with the Modified Hammersmith Functional Motor Scale, rather than relative fold change [15].

Evidence linking GAPDH directly to SMN can also be found from a previous highthroughput yeast two-hybrid screen [28], although (as with other protein candidates identified from similar high-throughput studies) it would appear that this has association has since been overlooked. Notably, the observation of reduced levels of GAPDH in SMA is consistent with a role as an SMN-interactor, since many other SMN-binding proteins are also dysregulated and/or differentially expressed in SMA models [14,20,29,30]. Although it has been widely implicated in cellular metabolic processes, GAPDH has also been linked to a range of

pathways with particular relevance to the nervous system and neurodegeneration, including apoptosis [31], and vesicle and axonal transport [32]. Documented variation in the mRNA and protein levels of GAPDH in other neuropathological events have been reported [33], and suggest that it may play a key role in neurodegenerative processes. Further investigations into the role of GAPDH in SMA pathogenesis therefore appear warranted.

In addition to implications for SMA pathogenesis, the significance of reduced GAPDH in SMA models should be viewed as a cautionary note, in so far that its use (as well as that of other house keeping proteins such as actin and tubulin) as an internal loading control for gene expression and western blot analysis may be unreliable [34]. As such, it may be necessary to revisit candidate proteins and genes from studies that have concluded little or no correlation with SMA in instances when levels were normalized to GAPDH (or other "house keeping" protein) expression.

Two other proteins whose levels were consistently reduced across three separate proteomic comparisons were growth-associated protein 43 (GAP43 or neuromodulin) and neural cell adhesion molecule (NCAM) (**Table 2**). Interestingly, both GAP43 and NCAM are known binding partners of the multi-functional calcium-binding protein calmodulin [35,36] – which itself features in **Table 2** as a down-regulated protein detected in two separate proteomic comparisons.

GAP43 levels were reduced by approximately 65% in iPS-derived SMA motor neurons [16], and by 35% and 22% in the SMA mouse hippocampus [18] and hippocampal synaptosomes [20] respectively (**Table 2**). Similarly, reduced levels of GAP43 have been detected in the CSF of human patients affected by a wide range of neurological disorders [37], including

motor neuron disease, movement disorders (e.g. Parkinson's disease) and multiple sclerosis. Considered to play a key role in axonal pathfinding, GAP43 is required for survival beyond early postnatal development in the mouse [38]. Reports have demonstrated increased levels of GAP43 during axonal growth in the rat [39] and during axonal regeneration following neurological injury of toad retinal ganglion cells [40] and the goldfish retina [41]. Very recently, reduced levels of GAP43 mRNA and protein in axons and growth cones of SMA mice were reported [42]. SMN appears to be responsible for regulating the localization and translation of GAP43 mRNA in these axons and restoration of GAP43 mRNA and protein levels by overexpression of the mRNA binding proteins, HuD and IMP1, rescue axonal growth defects in the SMA mice [42]. These studies illustrate that GAP43 is implicated in axonal growth / repair pathways and as such, represents a promising neuronal target for SMA therapy.

NCAM is a cell-surface protein that has been associated with cell–cell adhesion, neurite outgrowth and plasticity, and memory formation [43]. Maintenance of NCAM homeostasis is also important for neuronal growth and survival since both increased and decreased levels of NCAM have been linked to a wide range of neurological disorders (reviewed by Gnanapavan and Giovannonib [44]). For example, while levels of NCAM increase following axonal injury [45,46] and atrophic fibers in type I and II SMA muscle biopsies appear to express high levels of NCAM [47], NCAM deficient mice display hippocampal dysplasia and loss of septal cholinergic neurons [48]. Increased life-span and improved behavioural performance was seen in a mouse model of the motor neuron disease amyotrophic lateral sclerosis (ALS), following transplantation of human umbilical cord blood cells carrying adeno-viral vectors expressing a neuro-protective factor and NCAM [49]. It seems highly pertinent therefore, that future studies should aim to determine whether modulating the severity of SMA is also

possible by manipulation of NCAM levels, which until now, have been overlooked in terms of their role in SMA pathogenesis.

Of the 10 proteins that revealed increased expression in SMA cell and tissue samples, three were consistently changed across three separate proteomic comparisons (**Table 2**). Collagen VI, alpha 3 chain (COL6A3) was increased in SMA patient plasma [15] and in SMA patient fibroblasts and iPS-derived motor neurons [16]. Of particular interest is that when SMA fibroblasts were quantitatively compared to control fibroblasts, 18 proteins were differentially expressed but only one of these, collagen alpha-3 VI, was also differentially expressed in the same direction when genetically matched SMA iPS-derived motor neurons were compared to control motor neurons.

Over expression of COL6A3 is also seen in various types of cancer [50-52] and mutations in this gene can cause Ullrich congenital muscular dystrophy (CMD) and Bethlem myopathy [53]; both of which involve muscle and connective tissue problems. Though collagen VI is a well-known extracellular matrix protein, little is known about the particular characteristics of the COL6A3 isoform, except that it may play a role in advanced stages of neural crest development [54]. The relevance of an increase in the levels of COL6A3 in SMA may reflect an attempted protective response, since an increase in collagen VI appears to confer neuronal protection under cell stress [55].

Published interactors of SMN, annexin A2 (ANXA2) and the intermediate filament protein lamin A/C (LMNA) [56], were increased in expression in Schwann cells and muscle from SMA mice and in iPS-derived motor neurons from an SMA patient (**Table 2**). Mutations in LMNA cause a range of disorders, including muscular dystrophies such as Charcot-Marie-

Tooth disease and Emery-Dreifuss muscular dystrophy [57]. Given that mutations in the *LMNA* gene can cause an SMA phenotype [58,59], it is surprising that further studies to explore the relationship between LMNA and SMA have not been pursued.

Depletion of SMN in the NSC-34 mouse motor neuron cell line resulted in a 50% increase in the levels of ANXA2 mRNA [60] which correlates well with the 57% increase of ANXA2 protein seen in iPS-derived motor neurons from an SMA patient [16] (**Table 2**). Over-expression of ANXA2 by transient transfection induces the disassembly of Cajal bodies and relocalization of coilin [61], the "bridge" protein that mediates recruitment of SMN to the Cajal body [62]. This is particularly interesting in the current context, and may offer insight into the likely downstream consequences of increased ANXA2 in SMA cells, given that depletion of SMN also disrupts Cajal body formation and localization of coilin [63,64].

#### **Refinement of single quantitative proteomics datasets**

In addition to highlighting conserved changes present across several studies, review of multistudy proteomic comparisons are likely to be useful for refining single proteomics datasets by highlighting potential "false positives". Differentially expressed proteins that one might consider as "false positives" could arise due to unavoidable imprecisions in tissue sampling techniques, the impact of genetic diversity between the individual samples, the heterogeneous nature of some cell cultures, the developmental status of the samples, or differences in disease severity and progression. An example of the impact of the latter comes from our own previous work where we quantitatively compared the proteome of primary skin fibroblasts from an SMA patient with an unaffected SMA carrier. Myogenic cells present in the SMA fibroblast cell line but undetectable in the other resulted in an apparent increase in the myoblast-specific protein, desmin in the SMA cells [65].

Based on a combined review of raw data concerning differentially expressed proteins from the studies detailed in Table 1, 37 proteins were identified that appeared to have altered expression in one particular SMA cell type or tissue but with opposing detection in another. These contradictions are summarised in **Table 3**. If we were to speculate about these contradictions in the context of SMA, it would be parsimonious to suggest that some likely reflect a unique response of that particular system / cell type to reduced SMN, and could be useful for explaining the known disparity between vulnerabilities of distinct cell and tissue types in SMA [3]. However, "... nature seems unaware of our intellectual need for convenience and unity, and very often takes delight in complication and diversity" [66], and in reality, the complex diversity of changes observed between proteomic data sets may simply represent the highly complex biological systems being examined. Such systems are not 'static' and will represent cells, tissues and organs at different stages of differentiation, development, and disease time-course. Thus, the ability to compare data across multiple proteomic datasets generated from a range of tissue samples offers the possibility of refining the output of a single dataset in order to minimize the influence of such variables. The wealth of proteomic data now available in the public domain makes it possible for future studies of SMA to undertake such comparative assessments.

#### **Conclusions**

Proteomics techniques offer a powerful tool for obtaining disease-relevant mechanistic insights into SMA. By reviewing multiple proteomic datasets, it is possible to identify conserved molecular changes downstream of SMN occurring across a range of model systems. These changes likely represent "core" regulators of disease pathogenesis and, as such, may represent novel therapeutic targets. It will now be important to verify these

changes biochemically in a range of cells and tissues throughout disease progression and to determine whether their expression levels correlate with clinical severity. Therapeutic strategies for pharmacological or genetic manipulation of these molecules - or manipulation of upstream regulators of groups of molecules – offers the potential to complement strategies directed solely at SMN.

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#### **Conflict of interest**

The authors declare no conflict of interest.

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#### Figure 1: The SMN interactome

Ingenuity Pathway Analysis (IPA) software-based identification of all known interacting partners of SMN protein reported in the published literature. Candidate interactors are organised based on their predominantly reported subcellular localisation and does not mean that they are exclusively restricted to these positions. These interactions are direct (solid lines) or indirect (dotted lines), and may have been reported following identification at the genomic, transcriptomic or proteomic (DNA,RNA and/or protein) level. As a result, the schematic presented here represents the known "interactome" for SMN as identified by IPA.

### Table 1: Overview of SMA studies employing proteomic screens

The nine publications that have used unbiased proteomic comparisons to date for the identification of differentially-expressed proteins in SMA.

\*This data resulted from re-analysis of a previously published raw dataset [66].

| Species        | Sample type      | Differentially | Analysis<br>platform | Protein<br>database | Reference |
|----------------|------------------|----------------|----------------------|---------------------|-----------|
|                |                  | proteins       | plationin            | ualabase            |           |
|                |                  | <u>.</u>       |                      |                     |           |
| Human, SMA I.  |                  |                | iTRAQ; MALDI-        |                     |           |
| &              | Plasma           | 84*            | TOF/TOF (AB          | Unknown             | [15]      |
|                | iDSC motor       |                |                      |                     |           |
| Human SMA I    | neurons          | 98             | OTOF (AB Sciex       | Swiss-Prot          | [16]      |
|                | Skin fibroblasts | 18             | 5600)                |                     |           |
|                |                  |                | 2D-gels; ESI-        |                     |           |
| Human, SMA I   | Skin fibroblasts | 6              | QTOF                 | Unknown             | [17]      |
|                |                  |                | (unknown)            |                     |           |
|                |                  |                | Label-free; LTQ      |                     | X         |
| Mouse,         | Schwann cells    | 195            | Orbitrap Velos       | IPI-mouse           | [23]      |
| laiwanese      |                  |                | Pro (Thermo          |                     |           |
|                |                  |                | 2D-gels: MALDI       |                     |           |
| Mouse (normal) | NSC34 SMN-       | 7              | OTOF                 | Unknown             | [19]      |
| wouse (normal) | knockdown        | /              | (unknown)            | UIKIIUWII           |           |
|                |                  |                | iTRAQ; ESI-          |                     |           |
| Mouse, severe  | Hippocampus      | 39             | QTOF (Agilent        | IPI-mouse           | [18]      |
| SIVIA          |                  |                | 6520)                | <u> </u>            |           |
| Mouse severe   | Hinnocamnal      |                | iTRAQ; ESI-          | $\mathcal{C}$       |           |
| SMA            | synaptosomes     | 52             | QTOF (Agilent        | IPI-mouse           | [20]      |
|                |                  |                | 6520)                | <u> </u>            |           |
| Mouse, severe  |                  |                | Crhitran XI          |                     |           |
| SMA at P5      | Muscle           | 23             | (Thermo              | Swiss-Prot          | [21]      |
| (symptomatic)  |                  |                | Scientific)          |                     |           |
|                | Undifferentiated |                |                      |                     |           |
|                | embryonic stem   | 4              | 2D gols: MALDI       | NCBInr              | [22]      |
| Mouse, severe  | cells            | X              | TOF/TOF (AB          |                     |           |
| SMA            | Differentiated   |                | Sciex 4800)          |                     |           |
|                | embryonic stem   | 11             |                      |                     |           |
|                | cells            |                |                      |                     |           |
|                |                  | <b>C</b>       |                      |                     |           |
|                | C                |                |                      |                     |           |
|                |                  |                |                      |                     |           |
|                | V                |                |                      |                     |           |
|                |                  |                |                      |                     |           |

#### Table 2: The conserved molecular responses to reduced levels of SMN in SMA

Individual proteins that were differentially expressed across two or more separate comparisons are shown, along with the number of studies they

were identified in ("repeat hits"), the corresponding SMA model and any additional evidence for a role in the pathophysiology of SMA.

| Proteins with increased expression in SMA models |                          |   |   |
|--|--------------------------|---|---|
| Protein name                                     | Number of<br>repeat hits | SMA model   | Additional evidence in the pathophysiology<br>of SMA  |
| Annexin A2                                       | 3                        | Mouse Schwann cells [23]<br>Type I patient motor neurons [16]<br>P5 mouse muscle [21]                               | <ul> <li>Published interactor of SMN [56].</li> <li>Depletion of SMN in the NSC-34 mouse<br/>motor neuron cell line resulted in<br/>increased levels of ANXA2 mRNA [60].</li> </ul> |
| Lamin A/C  | 3                        | Mouse Schwann cells [23]<br>Type I patient motor neurons [16]<br>P5 mouse muscle [21]                               | <ul> <li>Published interactor of SMN [56].</li> <li>Mutations in the LMNA gene can cause<br/>an SMA phenotype [58, 59].</li> </ul>  |
| Collagen alpha 3 (VI)                            | 3                        | Type I patient skin fibroblasts [16]<br>Type I patient motor neurons [16]<br>Type I, II and III patient plasma [15] | -   |
| Gelsolin   | 2                        | Mouse Schwann cells [23]<br>Type I, II and III patient plasma [15]  | -   |
| Calreticulin                                     | 2                        | Type I patient motor neurons [16]<br>P5 mouse muscle [21]   | <ul> <li>Increased expression verified by<br/>western blotting in SMA mouse and<br/>patient muscles [21]</li> </ul>   |
| Tropomyosin 3                                    | 2                        | Mouse hippocampus [18]<br>Mouse embryonic stem cells (differentiated) [22]  | -   |
| ATP synthase subunits                            | 2                        | Mouse hippocampus synaptosomes [20]<br>Mouse Schwann cells [23]   | -   |
| Actin-related protein 2/3 complex                | 2                        | Mouse hippocampus synaptosomes [20]<br>Type I patient motor neurons [16]  | -   |

| Collegen alpha 1 () (I)         |   | Type I patient skin fibroblasts [16]             |                                   |
|---------------------------------|---|--|-----------------------------------|
| Collagen alpha 1 (VI)           | 2 | Type I, II and III patient plasma [15]           | -                                 |
|                                 | 2 | Mouse hippocampus synaptosomes [20]              |                                   |
|                                 |   | Mouse embryonic stem cells (differentiated) [22] |                                   |
| Plectin                         | 2 | Mouse Schwann cells [23]                         |                                   |
| Flectin                         | 2 | P5 mouse muscle [21]                             |                                   |
|                                 |   | Mouse Schwann cells [23]                         | SMN overexpression improves       |
| SOD1                            | 2 | Mouse binnocampus [18]                           | neuromuscular function and motor  |
|                                 |   | Mouse hippocatipus [10]                          | neuron survival in SOD1 mice [68] |
| Sorpin H1                       | 2 | Mouse Schwann cells [23]                         |                                   |
| Serbinuti                       | 2 | Type I patient motor neurons [16]                |                                   |
| Annexin A5                      | 2 | Mouse embryonic stem cells (differentiated) [22] |                                   |
|                                 |   | Mouse Schwann cells [23]                         |                                   |
| Filomin                         | 2 | Mouse Schwann cells [23]                         | NO                                |
|                                 |   | Type I patient motor neurons [16]                |                                   |
| Collagon alpha 1 (I)            | 2 | Mouse Schwann cells [23]                         |                                   |
|                                 |   | Type I patient motor neurons [16]                | -                                 |
| Collagon Joha 2 (I)             | 2 | Mouse Schwann cells [23]                         |                                   |
|                                 |   | Type I patient motor neurons [16]                | -                                 |
| Hypoxia up-regulated protein 1  | 2 | Mouse Schwann cells [23]                         |                                   |
|                                 |   | Type I patient motor neurons [16]                | _                                 |
| Glucose-6-phosphate isomerase   | 2 | Mouse Schwann cells [23]                         |                                   |
| Succese-o-phosphate isoffletase | 2 | P5 mouse muscle [21]                             |                                   |
|                                 |   | -0   |                                   |

| Proteins with decreased expression in SMA models |                          |  |   |
|--|--------------------------|--|---|
| Protein name                                     | Number of<br>repeat hits | SMA model  | Additional evidence in the pathophysiology<br>of SMA  |
| UBA1   | 3                        | Mouse hippocampus synaptosomes [20]<br>Mouse Schwann cells [23]<br>Type I patient motor neurons [16] | <ul> <li>Mutations in the UBE1 gene are<br/>associated with a rare X-linked infantile<br/>form of SMA [25,26].</li> </ul> |

|                               |   |   | • Experimental suppression of UBA1<br>phenocopied the SMA motor neuron<br>phenotype in zebrafish [20], and<br>phenocopied the Schwann cells defects<br>observed in SMA [23].                                       |
|-------------------------------|---|---|--|
| NCAM                          | 3 | Mouse hippocampus [18]<br>Type I, II and III patient plasma [15]<br>Type I patient motor neurons [16] | <ul> <li>Atrophic fibers in type I and II SMA<br/>muscle biopsies express high levels of<br/>NCAM</li> </ul>   |
| GAP43 (neuromodulin)          | 3 | Mouse hippocampus [18]<br>Mouse hippocampus synaptosomes [20]<br>Type I patient motor neurons [16]    | <ul> <li>Reduced levels of GAP43 mRNA and protein in axons and growth cones of SMA mice [42].</li> <li>Restoration of GAP43 mRNA and protein levels rescued axonal growth defects in the SMA mice [42].</li> </ul> |
| GAPDH                         | 3 | Mouse Schwann cells [23]<br>Mouse hippocampus [18]<br>Type I, II and III patient plasma [15]          | Published interactor of SMN (from yeast two-hybrid screen) [28]  |
| Heat shock protein 90B        | 2 | Mouse embryonic stem cells (differentiated) [22]<br>P5 mouse muscle [21]                              | -  |
| Calmodulin                    | 2 | Mouse hippocampus [18]<br>Type I patient skin fibroblasts [16]  | -  |
| Catalase                      | 2 | Type I, II and III patient plasma [15]<br>Type I patient motor neurons [16]                           | -  |
| Peroxiredoxin 2               | 2 | Mouse Schwann cells [23]<br>Type I, II and III patient plasma [15]                                    | -  |
| Aldehyde dehydrogenase        | 2 | Mouse embryonic stem cells (differentiated) [22]<br>Mouse Schwann cells [23]                          | -  |
| Alcohol dehydrogenase class 3 | 2 | Mouse Schwann cells [23]<br>Type I patient motor neurons [16]   | -  |

### Table 3: Proteins with contradictory expression levels in SMA models

A number of proteins appear to be increased or decreased in expression in a particular SMA cell type or tissue but showed the opposite trend in

#### another. (d) = differentiated.

| Proteins with contradictory expression levels in SMA models |   |   |
|---|---|---|
| Protein name  | Increased in SMA  | Decreased in SMA  |
| Aspartate aminotransferase                                  | P5 mouse muscle [21]  | Type I patient motor neurons [16]                             |
| Caldesmon   | Mouse Schwann cells [23]<br>Type I patient motor neurons [16]                           | Type I patient skin fibroblasts [16]                          |
| Catenin alpha   | Mouse Schwann cells [23]  | Type I patient motor neurons [16]                             |
| Catenin beta  | Mouse hippocampus synaptosomes [20]   | Type I patient motor neurons [16]                             |
| Endoplasmin   | Type I patient motor neurons [16]   | Mouse Schwann cells [23]                                      |
| Fructose bisphosphate aldolase                              | Mouse Schwann cells [23]<br>Mouse hippocampus synaptosomes [20]                         | Mouse hippocampus [18]  |
| Galectin  | Type I patient motor neurons [16]   | Mouse (NSC34) cells [19]                                      |
| Glyoxylase 1  | Mouse embryonic stem cells (d) [22]   | Type I, II and III patient plasma [15]                        |
| hnRNP K   | Mouse Schwann cells [23]  | Mouse (NSC34) cells [19]                                      |
| Hspa9/GRP75/Stress-70 protein                               | Mouse Schwann cells [23]<br>Mouse hippocampus synaptosomes [20]<br>P5 mouse muscle [21] | Mouse embryonic stem cells (d) [22]                           |
| HSP90a  | Mouse hippocampus [18]<br>Type I patient motor neurons [16]                             | Mouse hippocampus synaptosomes [20]                           |
| Hspd1   | Mouse Schwann cells [23]  | Mouse hippocampus [18]<br>Mouse hippocampus synaptosomes [20] |
| Inorganic pyrophosphatase                                   | Mouse hippocampus synaptosomes [20]   | Type I patient motor neurons [16]                             |

**Comment [A1]:** Author: there are two different table captions were provided, and this has been retained. Please check and confirm it is correct.

| Macrophage migration inhibitory factor   | Mouse hippocampus synaptosomes [20]  | Type I, II and III patient plasma [15]                          |
|--|--|---|
| MARCKS                                   | Mouse hippocampus synaptosomes [20]  | Mouse hippocampus [18]  |
| Myosin 3                                 | Type I patient motor neurons [16]  | Type I patient skin fibroblasts [16]                            |
| Park7                                    | Mouse hippocampus synaptosomes [20]  | Type I, II and III patient plasma [15]                          |
| Periostin                                | Type I patient motor neurons [16]  | P5 mouse muscle [21]  |
| Phosphoglycerate kinase 1                | Mouse hippocampus synaptosomes [20]  | Mouse Schwann cells [23]  |
| Phosphoglycerate mutase                  | Mouse hippocampus [18]   | Mouse hippocampus synaptosomes [20]<br>Mouse Schwann cells [23] |
| Profilin 1                               | Mouse Schwann cells [23]   | Type I, II and III patient plasma [15]                          |
| Prolyl 4-hydroxylase alpha 2 and alpha 1 | Type I patient motor neurons [16]  | Mouse Schwann cells [23]  |
| Reticulon                                | Mouse Schwann cells [23]   | P5 mouse muscle [21]  |
| Ribosome-binding protein 1               | Type I patient motor neurons [16]  | P5 mouse muscle [21]  |
| SOD1                                     | Mouse Schwann cells [23]<br>Mouse hippocampus [18]   | Type I, II and III patient plasma [15]                          |
| Sulfated glycoprotein 1                  | Mouse hippocampus synaptosomes [20]  | Mouse Schwann cells [23]  |
| Synaptopodin-2                           | Type I patient motor neurons [16]  | Type I patient skin fibroblasts [16]                            |
| S100 A4                                  | Mouse Schwann cells [23]   | Type I, II and III patient plasma [15]                          |
| T-complex protein 1                      | Mouse hippocampus synaptosomes [20]<br>Mouse Schwann cells [23]  | P5 mouse muscle [21]<br>Mouse hippocampus [18]                  |
| UCHL1                                    | Mouse embryonic stem cells (d) [22]<br>Mouse Schwann cells [23]<br>Type I patient skin fibroblasts [17]<br>Mouse hippocampus synaptosomes [20]<br>Type I patient skin fibroblasts [16] | Type I patient motor neurons [16]                               |
| VDAC1                                    | Type I patient skin fibroblasts [16]   | Mouse Schwann cells [23]  |
| Vimentin                                 | P5 mouse muscle [21]   | Type I patient motor neurons [16]                               |
| Zyxin                                    | Type I patient motor neurons [16]  | Type I patient skin fibroblasts [16]                            |
| 14-3-3 gamma                             | Mouse Schwann cells [23]<br>Mouse hippocampus synaptosomes [20]  | Mouse embryonic stem cells (d) [22]                             |

| 40S S3                                       | Type I patient motor neurons [16]    | Mouse Schwann cells [23]          |
|--|--------------------------------------|-----------------------------------|
| 2',3'-cyclic-nucleotide 3'-phosphodiesterase | Type I patient skin fibroblasts [16] | Type I patient motor neurons [16] |

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| eque o |       |
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