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A network-biology based approach for the development of SMN-independent treatments

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Abstract

Spinal muscular atrophy (SMA) is a devastating motoneuron (MN) disease caused by mutations or deletions of the Survival of Motoneuron 1 gene (*SMN1*), resulting in low levels of SMN protein. Current treatments for SMA, designed to restore SMN protein levels, have impressive effects but cause some side effects. Moreover, later stages of the neurodegeneration cannot be reversed only by SMN-restoration. It is, therefore, essential to better elucidate the molecular pathomechanism of the disease and to improve the current SMA therapeutic strategies, in order to significantly improve patients' lifestyle. In this regard, since SMN protein levels have already been enhanced in treated patients, complementary SMN-independent approaches are needed. To this purpose model organisms and bioinformatics are essential tools.

In SMA several altered signaling pathways regulating neurodegeneration have been identified. These signaling pathways form networks which connectivity is still not completely known. Elucidating the networks of altered signaling in SMA could be a good strategy to identify new potential SMN-independent therapeutic targets.

Using a phospho-array approach on SMA mice and network-biology, 38 dysregulated proteins involved in motoneuron degeneration have been identified, and B-Raf pinpointed as major signaling hub. Analyses in SMA mice revealed that B-Raf was down-regulated in motoneurons and in the spinal cord with B-Raf being reduced at pre-symptomatic stages. These results have been confirmed in patients and are also conserved across species; a *Caenorhabditis elegans* SMA model, indeed, showed less expression of the B-Raf homolog *lin-45*.

In this PhD project, the role of B-Raf/*lin-45* has been characterized *in vivo* using a *C. elegans* model of SMA, demonstrating its cell autonomous neuroprotective role when *smn-1* is down-regulated. This neuroprotective effect was demonstrated to be mediated by the MEK/ERK pathway. Then, using *C.elegans*, was investigated the role played by the others 37 proteins belonging to the network in order to choose the best target genes to be further studied. Thus, to rapidly identify modifier genes among those 37 candidates, a new *C. elegans* model of SMA has been set up. 16 new modifier genes among all the 37 genes tested have been identified. Using cell-specific RNAi, we demonstrated that 11 play a role in neurons and 5 of those specifically in MNs. Two of them, that act as suppressors of the neurodegeneration caused by *smn-1* down-regulation, have been chosen for further studies: (5 words omitted). Both are serine threonine kinases involved in several pathways overlapping with SMN function, thus, perfect candidates to be investigated. Although *C. elegans* has many experimental advantages, it is crucial to demonstrate that genetic interactions are conserved across species to make them more consistent (121 words omitted). These data unveil a neuroprotective role of a network of genes in MNs which are candidate targets for future therapies in SMA and support that the combined use of *in vivo* and *in vitro* model systems contribute significantly to achieving rapid progress in the study of a devastating disease such as SMA.

Introduction

CHAPTER 1

Spinal Muscular Atrophy

Spinal Muscular Atrophy (SMA) is a rare monogenic pediatric disease mainly affecting anterior horn motoneurons (MNs) of the spinal cord, resulting in progressive proximal muscle weakness and skeletal muscle atrophy. Symptoms appear at or shortly after births and are often fatal, making SMA one of the main genetic causes of infant mortality. The incidence of SMA is approximately 1 in 10,000 to 20,000 live births, and the carrier frequency is 1/40 to 1/70 in the general population (Nishio *et al.*, 2023).

1.1. Spinal Muscular Atrophy main features

Spinal Muscular Atrophy is a rare neuromuscular, neurodegenerative disease caused by mutations in the *SMN1* gene that result in a deleterious reduction of the survival motor neuron (SMN) protein. This causes dysfunction and neurodegeneration of α -motoneurons in the spinal cord and brain and, as a consequence, progressive muscle atrophy and weakness of the limbs, trunk, bulbar muscles controlling swallowing and respiratory muscles (Mercuri *et al.*, 2022).

According to the age of onset, the maximum motor function achieved and the survival of untreated patients, SMA is subdivided into a neonatal form (type 0), three pediatric forms (types 1-3) and an adult-onset form with milder phenotypes (type 4) (Mercuri *et al.*, 2022). Their main features are summarized in Table 1.1.

SMA Type	Age of onset	Maximal motor function achieved with supporting care	Feeding and Communication	Pulmonary function	Survival
0	Fetal	None	None	Very poor	Days-weeks
1	0 - 6 months	No sitting or rolling	Poor, require support	Poor, require support	Months (~10 months)
2	7 - 18 months	Sit, no walking	Variably affected	Reduced, often need support	Years (~20 years)
3	1.5 - 10 years	Limited walking	Normal	No symptoms, mild reduction possible	Normal
4	>18 years	Normal walking	Normal	Normal	Normal

TABLE 1.1. **SMA Classification:** Classification of SMA type according to main clinical features (adapted from Mercuri *et al.*, 2022)

Despite the most evident clinical manifestation of SMA, the skeletal muscle weakness, from the earliest stages of the disease SMA patients exhibit several defects affecting the nervous system both centrally and distally at the neuromuscular junction (NMJ). These include developmental defects affecting MNs cell body, axons and targeted myofibres, synaptic dysfunction and motoneurons hyper-excitability, motor axon radial growth, alterations in Schwann cell ensheathment and

myelination, reduced NMJ maturity and denervation (figure 1.1) (Ling *et al.*, 2010, Kariya *et al.*, 2008, Kong *et al.*, 2021).

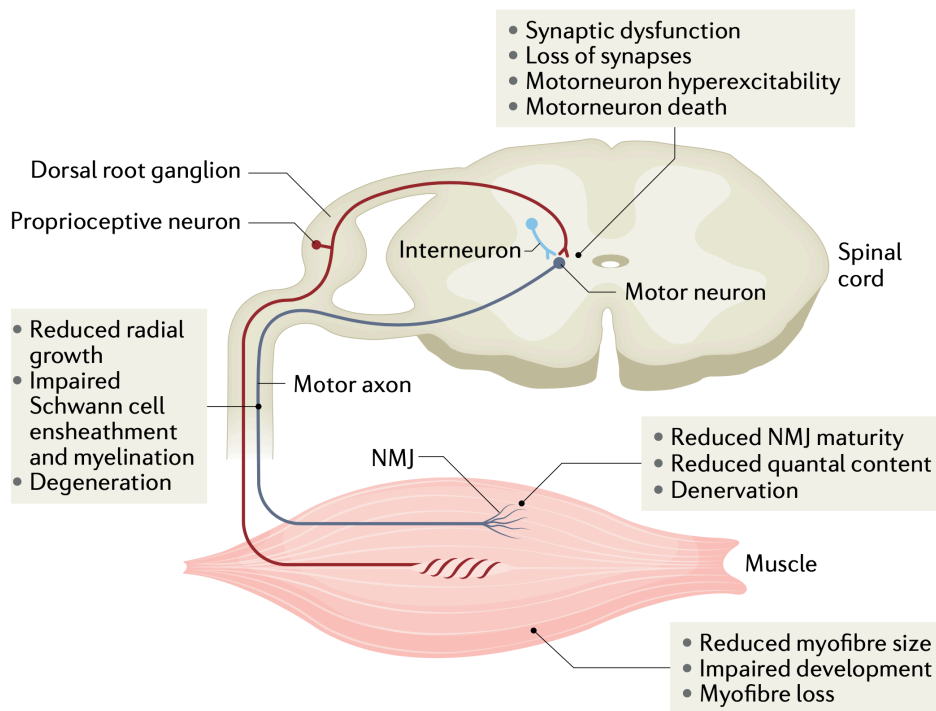


FIGURE 1.1. **Schematic representation of the key morphological defects in SMA:** In healthy condition, the proprioceptive neurons of dorsal root ganglion (in dark red) perceive the signal for muscle contraction and send synaptic inputs to motoneurons (in blue). MNs connect to the muscles via neuromuscular junction inducing muscle contraction through cholinergic neurotransmission. SMN deficiency disrupts this loop by altering neuronal function and muscle structure. The squares show the main morphological and functional defects observed in SMA motor system (Mercuri *et al.*, 2022).

Beyond its neurological features, growing evidence highlights the multi-systemic nature of SMA. Data from SMA patients and SMA mice, indeed, show dysfunction of skeletal muscle, heart, kidney, liver, pancreas, spleen, bone, connective tissues, and immune systems (Yeo and Darras, 2020).

1.2. Genetics of SMA

Spinal muscular atrophy is a monogenic autosomal recessive disease caused by homozygous deletions or loss of function mutations of the *SMN1* gene, resulting in a reduced expression of the survival motor neuron (SMN) protein. The SMA-causing gene locus has been mapped, in 1990, to the chromosome 5q11.1-13.3, a complex region containing a large inverted duplication. *SMN1* maps to the telomeric region, while its paralog gene, *SMN2*, maps to the centromeric region. Each *SMN* gene includes nine exons (Mercuri *et al.*, 2022). The *SMN1* and *SMN2* genes are nearly identical except for five nucleotide changes, one of which is a single nucleotide transition (coding variant c.840 C to T) in the exon 7 of *SMN2* (Lorson *et al.*, 1999). While *SMN1* pre-mRNAs are generally spliced to retain all the exons and encode functional full-length SMN proteins, the c.840 of *SMN2* inactivates an exon splicing enhancer creating an exon splicing silencer. This change is translationally silent, but results in an alternative splicing event in the exon 7 (Mercuri *et al.*, 2022).

As a consequence, about 90% of *SMN2* product is an mRNA isoform lacking of exon 7 and resulting in a truncated (*SMN Δ 7*) and unstable protein rapidly degraded; only about 10% of *SMN2*-derived transcript includes exon 7, resulting in low levels of full-length and functional SMN protein (figure 1.2) (Sendtner *et al.*, 2010). In healthy individuals, *SMN1* and *SMN2* are transcriptionally active and both stable and unstable SMN proteins are expressed. In contrast, SMA patients in whom *SMN1* is depleted or mutated, express only very low levels of stable survival motoneuron protein derived from *SMN2*; this amount is insufficient to fully compensate the *SMN1* loss and results in neuromuscular degeneration.

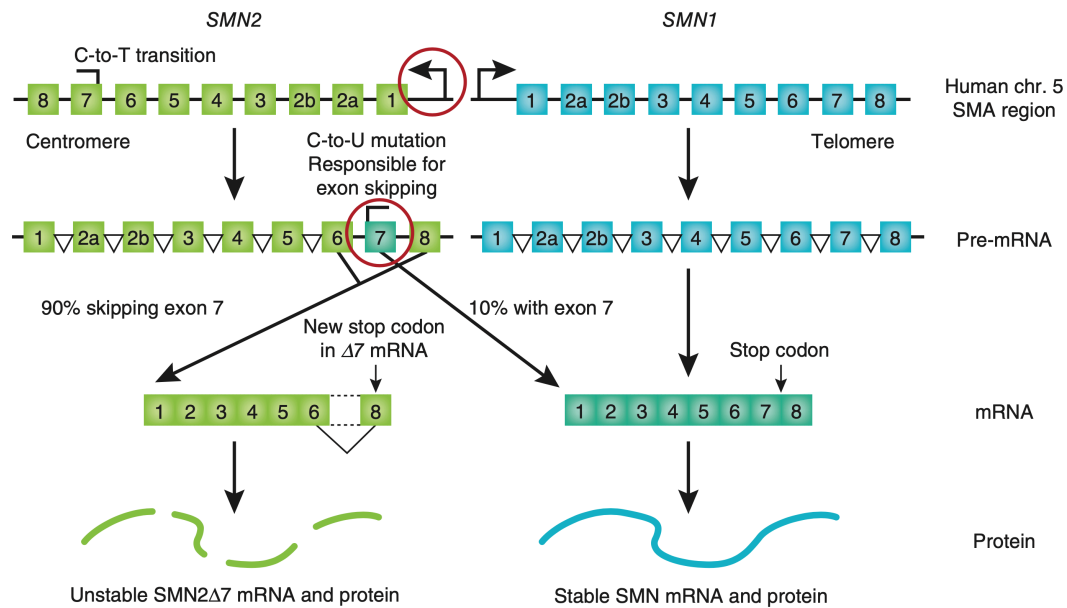


FIGURE 1.2. **Schematic diagram of *SMN1* and *SMN2* gene expression:** *SMN1* and *SMN2* share more than 99% nucleotide identity. Their main difference is a single nucleotide substitution in *SMN2*'s exon 7 (C to T). The transition causes the exon 7 skipping and the degradation of 90% of the *SMN2* protein (Sendtner, 2010).

Importantly, due to the instability of the human genomic region containing *SMN* genes (5q chromosomal duplication), *SMN2* copy number varies between individuals; this is crucial for SMA patients. *SMN2* copy number and phenotypic severity, indeed, are inversely correlated in patients. *SMN2* has been found to be a genetic modifier of SMA and patients with high *SMN2* copy number exhibit milder phenotypes demonstrating that the disease is due to the reduced amount of SMN protein rather than its complete absence (Tisdale and Pellizzoni, 2015).

1.3. Survival Motor Neuron Protein (SMN)

SMN genes encode the 38-kDa Survival Motor Neuron Protein (SMN), a 294 amino acid protein ubiquitously expressed that is localized to both cytosolic and nuclear compartments. SMN protein has been observed to localize in punctate structures in the nucleus, co-localizing with Coilin, a characteristic protein of Cajal bodies (Liu and Dreyfuss, 1996; Hebert *et al.*, 2001), which are subnuclear structures that play a role in mRNA metabolism; and it is also diffused in the cytoplasm co-localizing with sarcomeric Z-discs, microtubules, the Golgi network and cytosolic stress granules (Walker *et al.*, 2008; Ting *et al.*, 2012; Singh *et al.*, 2017).

The SMN protein contains multiple conserved domains: the N-terminal Gemin2 and nucleic acid-binding domains; a central Tudor domain; a C-terminal proline-rich and YG domains (figure 1.3).

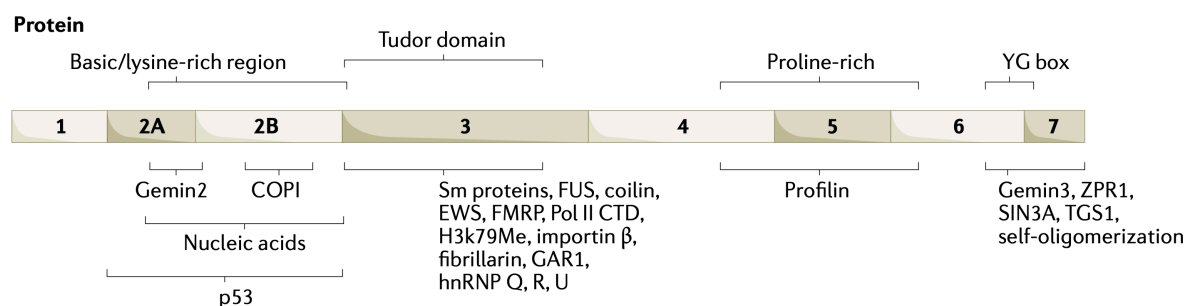


FIGURE 1.3. **Full-length SMN protein:** Schematic representation of full-length SMN protein. The relative size of the exons and the corresponding exon number is represented in the scheme. Each exon encodes a distinct functional domain (indicated above) which interacts with several proteins (indicated below) (Mercuri *et al.*, 2022).

Each domain has specific interacting partners and confers to SMN different functions (Singh *et al.*, 2017):

- The nucleic acid-binding domain, encoded by exons 2A-2B, overlaps with the binding site of the Gemin2 protein (Lorson *et al.*, 1998). The SMN-Gemin2 interaction is critical for several SMN functions, including snRNP assembly, DNA recombination, signal recognition, particle biogenesis and translation regulation. This domain also interacts with p53 a tumor suppressor protein and transcription regulator (Young *et al.*, 2002).
- The Tudor domain, encoded by exon 3, is involved in the interaction with proteins carrying RGG/RG motifs, such as hnRNPs and Sm proteins.
- The proline-rich sequence is encoded by exon 5. It interacts with Profilins, a family of proteins that control actin dynamics.
- The C-terminus YG box, encoded by exon 6, together with the last 16 amino acids, encoded by exon 7, are involved in self-protein oligomerization, which is critical for protein stability and protein localization.

The SMN protein is involved in multiple pathways and biological processes. The first identified and most studied role of SMN is its function in the assembly of the spliceosome and in the biogenesis of its major components, the uridine-rich small nuclear ribonuclear proteins (UsnRNPs). Thus, SMN is involved in the pre-mRNA splicing process following the interaction of UsnRNPs with unprocessed mRNA (Battle *et al.*, 2006; Chaytow *et al.*, 2018). In addition to its well-known role in the spliceosome, SMN has been shown to be localized in motoneuron neurites and growth cones, promoting the formation of complexes between RNA-binding proteins and mRNA to assemble various RNP particles involved in mRNA transport, metabolism and translation along the axon, as demonstrated by the presence of SMN in different macromolecular protein complexes in axons (Zhang *et al.*, 2006; Chaytow *et al.*, 2018). Accordingly, it has been demonstrated that, in the axonal compartments, SMN interacts with hnRNP-R and HuD, which bind and transport β-actin and acetylcholinesterase transcripts, respectively (Rossoll *et al.*, 2003; Chaytow *et al.*, 2018).

Neuronal architecture, function and development are also regulated by cytoskeletal rearrangements. Thus, SMN localization in neurites and growth cone, and its interaction with β -actin, suggests a further SMN role in the regulation of cytoskeletal dynamics. Indeed, growth cone formation and neurites outgrowth are mediated by actin dynamics, and SMN has been shown to interact with Profilin2a in both processes (Gieseemann *et al.*, 1999; Sharma *et al.*, 2005).

Additional SMN functions have been found in endocytosis, a basic cellular process essential for neuronal signalling and neuronal transmission, in autophagy processes, mitochondrial homeostasis, bioenergetics pathways and ubiquitination, underlying a possible role for SMN in protein homeostasis (Chaytow *et al.*, 2018).

1.4. Therapeutic strategies for SMA

Since the identification of the major causes of SMA in 1995, there has been a great progress in the design and the development of therapies for the disease. However, existing treatments for SMA are not feasible for all patients, often do not improve unrelated SMN phenotypes and cause undesired side effects. They, therefore, need to be improved. In this context two different therapeutic strategies to treat SMA can be identified: the SMN-dependent treatments, including the current drugs approved by the Food and Drugs Administration (FDA) and European Medical Agency (EMA); and the SMN-independent therapies which are currently developed in the pipeline.

1.4.1. SMN-dependent treatments

SMN-dependent treatments aim to increase SMN protein levels by two main strategies: modifying *SMN2* gene expression and *SMN1* gene therapy.

The first drug approved by the FDA and EMA was Nusinersen (Spinraza®; Biogen, Cambridge, MA, USA). It is an Anti-Sense Oligonucleotide (ASO) drug that targets the Intron Splicing Silencer (ISS) in *SMN2* intron 7, ISSN1, modulating the *SMN2* alternative splicing and promoting the retention of exon 7 in *SMN2* mature transcript. This allows *SMN2* to encode a full-length and stable SMN protein (figure 1.4) (Ottesen, 2017) .

Since ASOs don't cross the blood-brain barrier, Nusinersen is administered by intrathecal injection three or four times over two months during the initial loading period and every four or six months during the maintenance period (Nishio *et al.*, 2023). Although Nusinersen was the first drug to be approved and shows promising and effective effects improving significantly patients' motor functions and survival, there are some limitations to its use. First, data from clinical trials suggest the importance of a therapeutic window when increasing of SMN levels are more effective; secondly, the intrathecal administration is an invasive procedure, and the need for regular lumbar injection increases several risks, including exacerbation of respiratory compromise related to the knee-to-chest flexion posture during the procedure, headache and cerebrospinal fluid leakage; third, as Nusinersen is only delivered in the spine, SMN up-regulation is mainly restricted to the Central Nervous System and not to peripheral tissues (Chen, 2020).

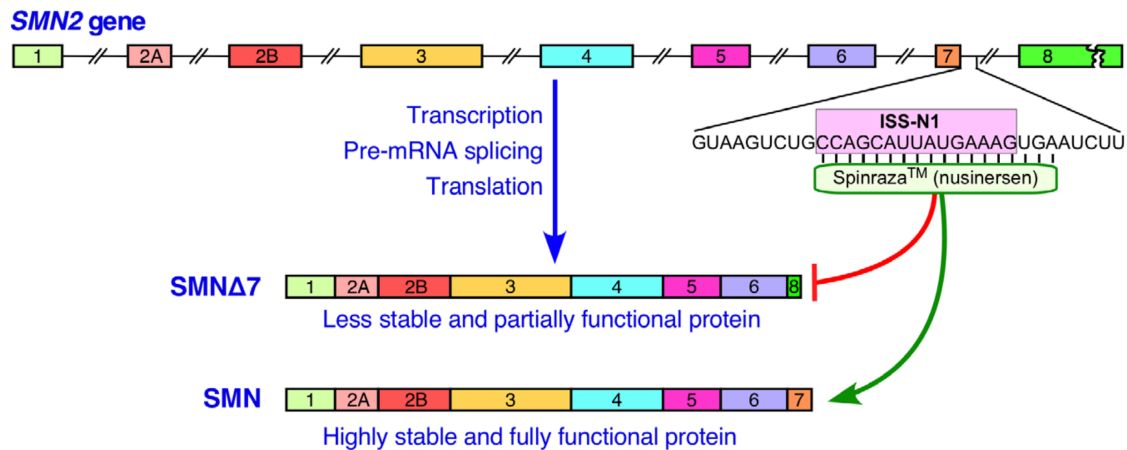


FIGURE 1.4. **Molecular mechanism of Nusinersen:** the picture shows an overview of *SMN2* gene and the mechanism of action of Nusinersen. Colored boxes are exons, broken lines are introns. Region of ISS-N1 is shown. ISS-N1 annealing location of Nusinersen is indicated. Protein products of *SMN2* are shown. Nusinersen acts by redirecting splicing from the unstable product to the stable full-length protein (Ottesen, 2017).

Among the strategies aimed at modifying *SMN2* gene expression, FDA approved Risdiplam (Evrysdi®; Roche, Basel, Switzerland) in 2020 and EMA the following year (Ratni, 2019). Initially approved only for the treatment of SMA patients older than 2 months of age, Risdiplam is now also approved in the USA for pediatric and adult patients. Risdiplam is an oral solution containing a small molecule compound that, as Nusinersen, acts as *SMN2* splicing modulator. Unlike Nusinersen, Risdiplam binds to two sites on the *SMN2* mRNA, the Exonic Splicing Enhancer 2 (ESE2) and the 5' splicing site of exon 7. This interaction stabilizes the ribonucleoproteins complex promoting exon 7 inclusion and the translation of full-length SMN protein (figure 1.5). Risdiplam is taken orally once per day, making it a non-invasive treatment that doesn't require hospitalization.

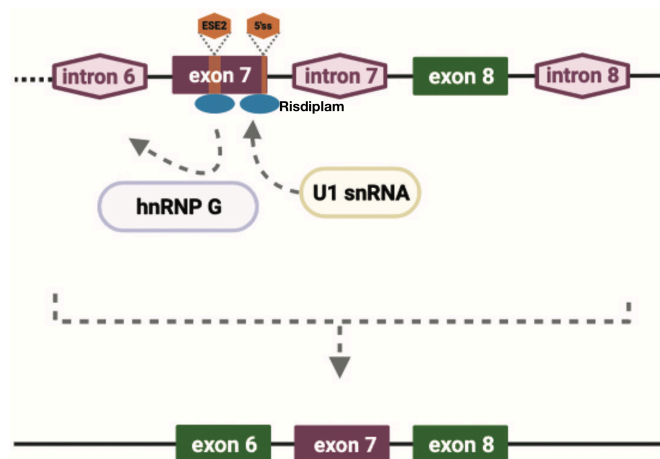


FIGURE 1.5. **Risdiplam mechanism of action:** the picture shows a schematic representation of Risdiplam mechanism of action. The compound binds on two sites within the exon 7 of the *SMN2* transcript, namely exonic splicing enhancer 2 (ESE2) and 5' splice site (5'ss). Binding to the 5'ss enhances the binding of the U1 snRNA. The interaction with the ESE2 is believed to lead to dislocation of the hnRNP G allowing the binding of the U1 snRNP complex. These changes ultimately lead to the inclusion of exon 7 and the production of a full-length *SMN2* mRNA (Markati *et al.*, 2022)

The third treatment for SMA patients is Onasemnogene abeparvovec (Zolgensma®; Novartis Gene Therapies, Bannockburn, IL, USA). It is the second FDA- and EMA- approved treatment for SMA, introduced in clinical practice after Nusinersen. Onasemnogene abeparvovec follows a gene therapy strategy aimed at reintroducing the full *SMN1* gene in patients. It is an adeno-associated virus (scAAV9) vector-based drug able to cross the blood–brain barrier and a drug available for all SMA patients with all type of SMA who are younger than two years old at the time of dosing. The AAV9 vector carries the cDNA for the expression of the full-length SMN protein. Upon entry in the host cell, AAV9 translocates in the nucleus where the carried transgene acts as an episome, expressing a stable and completely functional SMN protein (figure 1.6) (Nishio *et al.*, 2023).

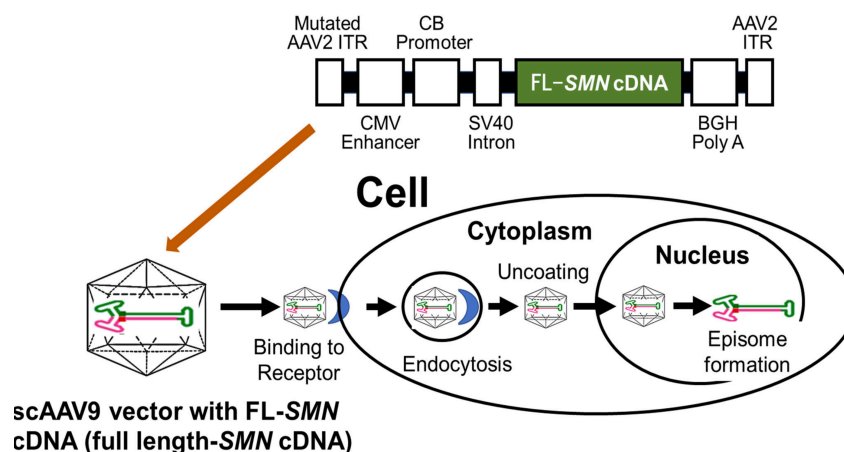


FIGURE 1.6. **Mechanism of action of onasemnogene abeparvovec** : the picture shows a schematic representation of Onasemnogene abeparvovec transgene structure and its mechanism of action mediated by AAV9. Above is represented the double strand transgene expressing the full-length *SMN* cDNA. Upstream of cDNA is a AAV2 inverted terminal repeat (ITR) able to speed-up the transcription of the double-stranded transgene; it is followed by the hybrid CMV (cytomegalovirus) enhancer and chicken β -actin (CB) promoter that activates the transgene to allow for continuous and sustained SMN protein expression. After entering the host cell, the scAAV vector translocates into the nucleus (picture below), where the transgene acts as an episome, expressing the full-length SMN protein (Nishio *et al.*, 2023).

Although gene therapy should be a definitive and completely resolutive treatment, Onasemnogene abeparvovec has limitations: firstly, its high cost, approximately €1.9 million per course of treatment (Nuijyen, 2022); secondly, the limited time window of administration, which require an early diagnosis of the disease; third, serious adverse events can also occur, including transient hepatotoxicity and thrombotic microangiopathy (Nishio *et al.*, 2023).

SMN-dependent treatments significantly improve motor function and lifestyle in SMA patients. However, their high cost, the undesired side effects they can cause, the still unclear long-term effect of an AAV9- mediated over-expression of SMN, and the need of treating also the later stages of the neurodegeneration, that can't be reversed only by SMN-restoration, highlight the importance of developing new therapeutic strategies that can improve and complement the existing ones; in this scenario the development of SMN-independent treatments is crucial.

1.4.2. The need of new therapeutic strategies and how network biology could contribute in this context

Although SMN protein deficiency mainly affects neuronal and motor functions in SMA patients, due to its ubiquitous expression, SMN lack can also affect other systems, pathways, and processes, such as metabolic alterations, muscle, heart, vasculature, pancreas and liver (Hensel *et al.*, 2020). In this scenario, the SMN-dependent treatments, that restore SMN levels in central nervous system (CNS), should be complemented with new therapeutic strategies able to restore SMN systemically. Importantly, the beneficial effect of actual treatments are strongly dependent from the diagnosis, and the time of intervention. This and the occurrence of non-responders patients, suggest the presence of pathological mechanisms that can't be reversed only by enhancing SMN protein levels (Hensel *et al.*, 2020).

In this scenario, SMN-independent treatments should include both, therapeutic strategies that do not increase directly SMN protein levels; or therapeutic approaches focused on treating new SMN-irreversible targets.

SMN-independent strategies include (*curesma.org*):

- Muscle protection to prevent or restore the loss of muscle function, such as small molecules that enhance the muscle's ability to contract, or regulators of muscle mass that may improve muscle strength, or molecules able to improve the function of NMJ;
- Neuroprotection of the motoneurons affected by the loss of SMN protein, by restoring their function or preventing their death. This includes the identification of new small neuroprotective molecules such as Riluzole (Russmann *et al.*, 2003) or natural compounds as green kiwi fruit (Mazzarella *et al.*, 2019);
- Approaches that identify additional systems and pathways affected by SMA to identify new molecular modifier that can be targeted to complement the actual approved treatment.

Regarding the identification of new systems and pathways affected in SMA, network biology could offer a comprehensive approach to understand the interactions between proteins involved in signaling pathways. These interactions are showed as connection between network nodes (the proteins). One of the key applications of network biology in signaling is the identification of critical nodes, known as hubs, that act as master regulators of cellular processes. These highly connected hubs could play a role in coordinating and controlling signaling cascade, making them attractive drug targets. Additionally, inter-modular nodes, which connect distinct signaling modules, can have even broader regulatory impacts, potentially influencing multiple cellular processes simultaneously. In SMA field, network biology provides important information on the molecular alterations that underlie the pathological condition. This can contribute to elucidate the pathomechanism of the disease, and to develop novel therapeutic strategies that target the causes of disease rather than individual symptoms (Hensel *et al.*, 2020).

In this scenario, Prof. Claus group, with whom a prolific collaboration was born some years ago, designed a phospho-array followed by a network biology approach that allowed to identify a new SMA modifier, that has been then characterized. Part of the results of this collaboration are described in this thesis and were published in 2021 (Hensel *et al.*, 2021).

1.5. Dysregulated signaling pathways in SMA spinal cords form a network with B-Raf in its center

Hensel *et al.*, designed a new experimental approach for a global and unbiased detection of altered signaling at pre-symptomatic stages. A severe Taiwanese SMA mice model has been employed. Postnatal day 1 (P1) and 3 (P3) animals were included for the detection of pre-symptomatic changes and P5 animals to confirm that alterations persist at the onset of the disease. The lumbar spinal cords were screened with an array containing over 1,300 antibodies able to detect and quantify non-phosphoproteins and phosphoproteins involved in diverse signaling pathway. 38 proteins that became differentially expressed or phosphorylated between SMA and control conditions in postnatal development have been identified. Next, they have performed a gene ontology (GO) enrichment analysis for a global view on the signaling pathways altered in SMA mice. GO analysis revealed two major pathways which were linked to dysregulated signaling in SMA: the PI3K/AKT signaling as well as MAPK signaling with an emphasis on Raf kinases. Signaling pathways often form cascades where an upstream kinase phosphorylates a downstream target, and a dysregulation of a signaling protein may induce a coregulatory pattern in its downstream targets. Therefore, the dysregulated proteins were subjected to a hierarchical clustering and a network analysis. The network forms two main clusters connected by B-Raf as the central inter-cluster node (figure 1.7) (Hensel *et al.*, 2021).

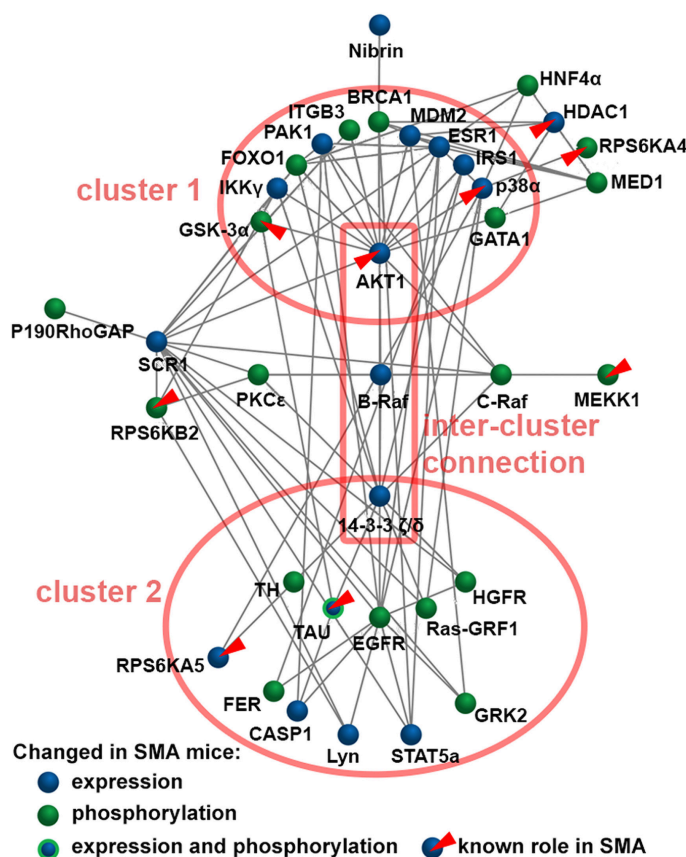


FIGURE 1.7. **Network analysis and identification of a new signalling hub:** Network analysis of altered proteins in SMA mice which are connected by edges based on known interactions from the Biogrid database. The connectivity determined the topology of the network. Nodes differentially expressed are the blue circles, the ones differentially phosphorylated are green. Blue nodes with green borders are proteins both differentially expressed and phosphorylated. Nodes which have been previously determined to be involved in SMA pathogenesis are highlighted with red triangles (Hensel *et al.*, 2021).

B-Raf is a member of the Raf-family serine/threonine kinases and an integral part of the Ras-Raf-MEK-ERK signaling axis. It is essential for survival of motoneurons in response to neurotrophic factors. The array data revealed a developmental dysregulation of B-Raf being down-regulated in SMA mice at pre-symptomatic P3 and at onset P5. These results have been confirmed *via* immunoblotting.

A reduced expression may not necessarily result in a reduced B-Raf activation. B-Raf is activated by a direct interaction with Ras at the plasma membrane. This leads to a conformational change releasing the auto-inhibitory domain and to phosphorylation at N-terminal residues (Lavoie and Therrien, 2015). Phosphorylation on Serine-445 by the casein kinase 2 reduces the level of auto-inhibition and is critical for B-Raf activation (Tran *et al.*, 2005). Thus, the total levels of B-Raf phosphorylated at Serine-445 have been evaluated. According to expressional reduction, the level of phosphorylated B-Raf was significantly reduced in the spinal cord of SMA mice compared to control (figure 1.8). Therefore, in SMA, B-Raf is not only less expressed but also less active (Hensel *et al.*, 2021).

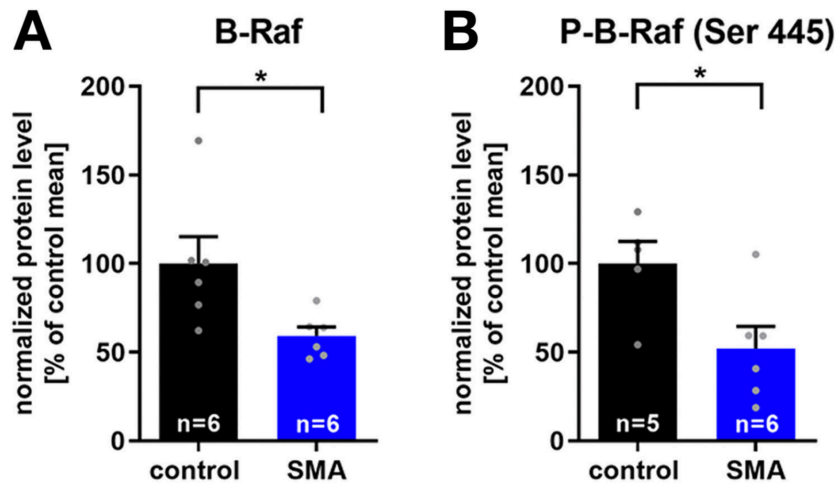


FIGURE 1.8. **B-Raf and phosphoB-Raf protein levels:** the picture shows B-Raf (A) and P-B-Raf (B) regulation in P5 control (black bars) and SMA mice (blue bars). Both B-Raf and P-B-Raf protein levels are strongly reduced in SMA condition (Hensel *et al.*, 2021)

These results have also been confirmed in patients, where B-RAF and phospho-B-RAF protein levels have been evaluated in cultured primary fibroblasts from severe SMA type I and SMA type 0 patients as well as from healthy control, and in induced pluripotent stem cells (iPSCs) differentiated to motoneurons obtained from SMA type I patients and healthy controls (Hensel *et al.*, 2021).

Starting from this preliminary result, we collaborated with Prof. Claus to first demonstrate the role of B-RAF in neurodegeneration *in vivo* and then to investigate the whole network of potential SMA modifiers. For this purpose, we used a small model organism, the nematode *Caenorhabditis elegans*.

CHAPTER 2

Model systems to study SMA

The rapid advances in SMA research and investigation would not have been possible without the wide range of SMA model systems and animal models. These include mice and patients-derived cell lines, vertebrate model organisms such as mice, and invertebrate model organism such as *Caenorhabditis elegans*, which will be discussed in the next chapter. At first, it is described the mammal model systems which I have used in this PhD project.

2.1. An *in vitro* model to study Spinal Muscular Atrophy: NSC34 cell line

In vitro models play a crucial role in advancing the understanding of SMA pathogenesis and identifying new potential therapeutic targets. These can include immortalized mice cell lines, such as NSC34 cells.

NSC34 cells are a hybrid mouse motoneuron (MNs) cell line derived from the fusion of motoneurons-enriched embryonic mouse spinal cord cells and mouse neuroblastoma cells (Cashman *et al.*, 1992). Cultures of NSC34 cells contain two distinct cellular populations: small undifferentiated cells capable of undergoing cell division, and multi-nucleated cells. NSC34 cells express many MNs properties, including the expression of choline acetyltransferase, the ability to release acetylcholine and to generate an action potential (Cashman *et al.*, 1992).

In 2017, G. Viero's group, using CRISPR-Cas9 technology, developed a motoneuron-like cell line (NSC34) expressing different levels of SMN protein. Particularly, following CRISPR-Cas9 technology they performed a stable SMN knock-out in NSC34 cells and isolated three independent cell lines expressing wild-type (as control), 20% or 0% of full-length SMN (Bernabò *et al.*, 2017).

2.2. A SMA animal model: Taiwanese SMA mice

The main limitation of model organisms is that none of them harbor the *SMN2* gene which ensures a constant low level of SMN protein, reducing *SMN1* deficiency deleterious effects (Bebée *et al.*, 2012). Homozygous knock-out of *SMN-1* in model organisms results in embryonic lethality. This is the reason why modeling SMA in animals is a real challenge. Nevertheless, mice have been widely accepted as model organism to study SMA. Humans and mice have highly conserved composition and organization of the nervous system and the structure and the function of SMN are conserved in these animals, as well. As with all model organisms, modeling SMA in mice is complicated because humans have two *SMN* genes while mice have only one: *Smn*. This murine gene encodes almost exclusively full-length transcripts, analogous to human SMN1. Similar to humans, complete SMN protein deficiency in mice is lethal. Therefore, *Smn* knock-out generates inactive Smn protein and results in lethality at the 8-cell stage of development (Bebée *et al.*, 2012). To create a mouse exhibiting an SMA disease phenotype, as well as to mimic the mechanisms of disease, human

SMN2 has been incorporated into *Smn* null mice. A commonly used mouse model in SMA field is the Taiwanese mouse. These animals are homozygous for the murine knockout allele (*Smn*^{-/-}) and heterozygous for a transgenic allele with two human *SMN2*-copies (Hsieh-Li *et al.*, 2000). Newborn mice are indistinguishable from wild type animals, but by P2 (post-natal day 2) they begin to develop SMA phenotypes such as reduced size, abnormal neurofilament protein accumulations in presynaptic motoneurons and progressive weakness; moreover, *Taiwanese SMA* mice lost among 40% of the motoneurons in the spinal cord by 3 post-natal days (Hsieh-Li *et al.*, 2000). These animals rarely live beyond P4 and P6 (Park *et al.*, 2010). Since the introduction, in the early 2000's, of the mice SMA models lots of improvement have been made in the knowledge of both biological and genetics aspects of SMA, and also they have brought many advantages in the therapeutic research for medical treatments. On the other hand, this model can't answer all questions we have on this disease, for example is not certain when during the course of the disease SMN must be restored to produce full phenotypic rescue; moreover it isn't clear whether a requirement for the protein applies equally to all periods of postnatal development or whether there are developmentally vulnerable and/or refractory periods. To answer those questions further investigation, through more complex models, might be required (Park *et al.*, 2010).

Although cells and mice models have allowed significant progresses in SMA research, they have limitations, primarily due to the increasing difficulty of performing *in vivo* analyses and obtaining data about the whole organism, as well as the time required to obtain such results. In this context, is becoming increasingly clear that there is a need to complement their use with other model organisms that are less ethically constrained, while still complying the 4Rs principles for animal research (Replacement, Reduction, Refinement and Responsibility/Reject) (Curzer *et al.*, 2016), and allowing *in vivo* analysis: *Caenorhabditis elegans* can help in this regard.

CHAPTER 3

Caenorhabditis elegans

Caenorhabditis elegans is a non-parasitic roundworm nematode, introduced by Sidney Brenner in 1960s as a genetic model to study development and nervous system. Since then, this nematode has become a model organism increasingly spreading in the scientific community for studies on developmental biology and neurobiology. It was the first multicellular organism to have its entire genome sequenced, and it is the only adult organism to have the whole connectome mapped (Cook *et al.*, 2019).

3.1. Introduction on *C.elegans*

Caenorhabditis elegans is a small (approximately 1mm) transparent free-living nematode found worldwide. Like all nematodes, it has almost 1000 cells, a cylindrical shape with bilateral symmetry and a collagenous cuticle. Even though *C. elegans* is considered a simple system, it shares many similarities with more complex organisms: 60-80% of homology with human genes, complex anatomical structures, such as the nervous system, a rich array of sophisticated behaviors, including social, sexual and learning behaviors (Corsi *et al.*, 2015). All these features make *C. elegans* a powerful tool for modeling human disorders.

C. elegans is a diploid animal whose karyotype consists of 5 pairs of autosomal chromosomes and one pair of sexual chromosomes. The nematode has a rapid life cycle (3-4 days at 20°C from egg to egg-laying adult) and exists as two sexual forms: a self-fertilizing hermaphrodite (genotype XX), and males (genotype XO) that arise at a frequency of <0.2% and are essential for genetic variability.

3.1.1. *C.elegans* life cycle

C. elegans embryogenesis lasts approximately 14 hours at 20°C, 9 of which are *ex utero* development. An impermeable eggshell and yolk granules are made after fertilization, allowing the embryo to develop independently of the mother. Embryos are usually retained within the hermaphrodite until the 24-cell stage. The hermaphrodite embryo hatches with almost 600 cells and becomes a first stage (L1) larva. The animals begin to eat and develop through the four larval stages (L1-L4) (figure 3.1) (Corsi *et al.*, 2015). Approximately 18 hours after the L4 stage, adult hermaphrodites begin to produce a progeny for 2-3 days until they have used up all their self-produced sperm; progeny can also be generated when the hermaphrodite mates with a male. After the reproductive period, hermaphrodites can live several more days before dying of senescence.

Although 20°C is used as reference temperature for nematode maintenance, *C. elegans* can easily grow at different temperatures, with physiological growing condition between 15°C and 25°C.

Temperature profoundly affects life cycle and aging. Indeed, worms that grow at lower temperatures show an extension of their lifespan, while animals that grow at higher temperatures have a shorter one. Particularly, under favorable conditions animals need 2.5 days to complete the

life cycle at 25°C. During development, individual sexes are not easily distinguished until the L4 stage. At the L4 stage, hermaphrodites have a tapered tail and the developing vulva is a half-circle in the center of the ventral side, while the males have a wider tail than hermaphrodite. In adults, the two sexes can be distinguished because males have a slim girth and fan-shaped tail. Sperm from the hermaphrodite or sperm obtained from males through mating can fertilize oocytes (Corsi *et al.*, 2015).

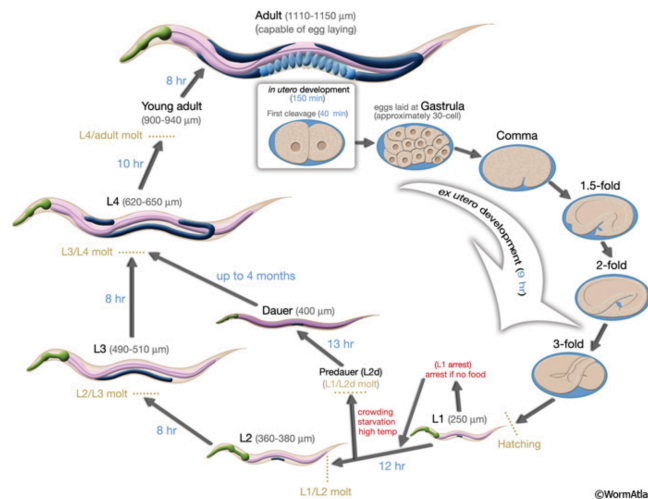


FIGURE 3.1. *Caenorhabditis elegans* life cycle: the picture shows *C. elegans* life cycle. Under favorable conditions animals pass through direct development to adulthood in ~5 days at 15°C, ~3 days at 20°C, ~2.5 days at 25°C. In response to harsh environmental conditions, such as food shortage, crowding or high temperatures, animals can enter an arrested dauer stage (www.media.springernature.com).

3.1.2. Main advantages of *C. elegans* as model organism

C. elegans is a powerful tool for studying eukaryotic biology. It has many advantages as a model organism, including: small size; large brood size; ease to cultivate; low maintenance expenses; long-term cryopreservation; quick generation time (less than a week at 20°C and 25°C); transparency, which allows researchers to perform *in vivo* experiments using fluorescent protein reporters, like GFP and RFP; the ability to reduce gene activity through RNAi by feeding; or, as many genetic models, the ability to finely modulate transgene expression (Corsi *et al.*, 2015).

Furthermore, although the worm presents less limitations compared to more complex organisms, as well as less ethical concerns compared to small vertebrates and mammals, it provides insights into the understanding of many fundamental mechanisms underlying biological processes, that have to be shown to be conserved in mammals, including humans. All these features make *C. elegans* a powerful tool for modeling human diseases, performing genetic studies, modulating gene expression, and investigating the biological mechanisms that cause the diseases.

3.1.3. Modulating gene expression: heat-shock

One of the main advantages of the genetic tools in *C. elegans* is the possibility to control transgene expression, in order to study not only the effect of gene activation or inactivation, but also temporal aspects of transgene expression. One of the most efficient strategies that allows a temporal control of transgene expression is provided by the defense mechanism to temperature stress, the heat-shock

related gene activation. This mechanism is mediated by heat-shock transcription factors that are synthesized constitutively but remain latent until a heat-stress happens, such as heat increase. In response to heat stress, heat-shock transcription factors are activated and they bind with high affinity specific promoters leading to the transcription of heat-shock proteins. Therefore, a transgene under control of a heat-shock promoter can be easily induced following a temperature shift (Bacaj and Shaham, 2007).

In *C. elegans* the most used heat-shock promoters are *hsp-16.2* and *hsp-16.41*: the first one promotes strong expression in hypodermal cells and neurons, while the second one is more efficient in directing expression in the intestine and pharyngeal tissues (Stringham *et al.*, 2013).

3.1.4. Modulating gene expression: RNA interference (RNAi)

Since its discovery in 1998 by A. Fire and C. Mello (Fire *et al.*, 1998), RNA interference has become an established method for studying gene function and gene interactions and has been the basis of several genome-wide screens, mainly due to its efficacy, specificity, speed and simplicity (Conte *et al.*, 2017). In *C. elegans*, RNAi can be performed by delivering double-stranded RNA (dsRNA) corresponding to a specific gene, following different strategies such as microinjection, feeding or soaking (Conte *et al.*, 2017); or by inducing the *in vivo* transcription of dsRNA by driving its expression in subset of cells when sense and antisense transgenes are co-injected (Esposito *et al.*, 2007). The choice of the RNAi strategy strongly depends on the experimental goals and needs; in this context, a good and fast method to silence a large number of genes is to set-up a RNAi *by feeding* screening using a library of RNAi clones. RNAi *by feeding* consists in using engineered bacteria carrying a plasmid expressing the dsRNA against the gene of interest, as source of food. As the worms eat and process the bacteria, the dsRNA is released and the RNAi machinery is induced (figure 3.2) (Grishok, 2005).

Despite its widespread use, RNAi has some limitations, mainly related to its efficiency. Indeed, some tissues, including the vulva, sperm and nervous system, are refractory to this process (Conte *et al.*, 2017). The reasons for this are not fully understood, but could include inefficient maintenance of the trigger dsRNA. Moreover, in the nervous system, *eri-1*, an exonuclease that antagonizes RNAi by degrading siRNA, is highly expressed (Grishok, 2005).

Since *C. elegans* is a powerful tool for studying neurobiology, improving RNAi in the nervous system has been a major challenge. A good strategy is to manipulate the RNAi machinery. In particular, the double mutant *eri-1(mg366); lin-15b(n744)* has been largely used to enhance RNAi sensitivity in all the body, including nervous system (Vashlishan *et al.*, 2008).

Once delivered, the dsRNA spreads throughout body to produce a systemic RNAi effect. This mechanism is mediated by specific pathways involving multi-pass transmembrane proteins, such as *sid-1*, which allows dsRNA to spread in neighbor cells (May and Plasterk, 2005). Although spreading is a critical event to increase RNAi efficiency, it could be limiting when the gene of interest is essential, or expressed in all tissues and the function in a particular tissue is being investigated, such as a specific subclass of neurons. To avoid this issue, tissue-specific RNAi strains have been developed. These strains are RNAi-deficient mutants expressing tissue-specific

transgenes that compensate for the RNAi-deficiency. For instance, in 2013, Firnhaber and Hammarlund developed a GABA MNs-specific RNAi strain. This strain harbors a *rde-1* loss of function mutation that causes systemic RNAi resistance, specifically rescued in MNs by the expression of a transgene encoding *rde-1* and *sid-1*, that promotes dsRNA uptake to motoneurons (Firnhaber and Hammarlund, 2013).

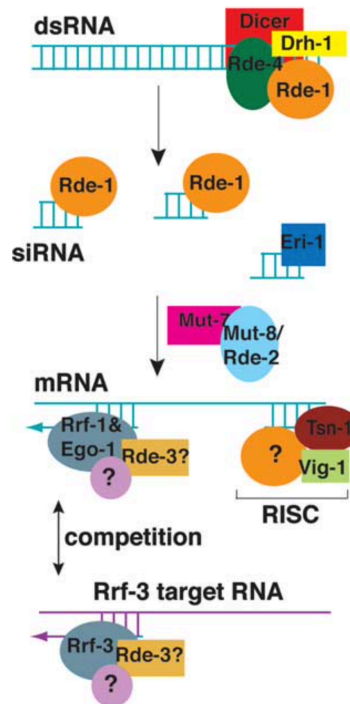


FIGURE 3.2. **Schematic representation of RNAi mechanisms in *C. elegans*:** Exogenous dsRNA is processed into siRNAs by the Dicer complex containing: Dicer (red), the dsRNA binding protein RDE-4 (green), the PAZ-PIWI protein RDE-1 (orange) and the Dicer related helicase DRH-1 (yellow). RDE-1 binds the siRNAs, bringing them to the next step in the RNAi pathway. ERI-1 (blue) antagonizes RNAi by degrading siRNAs. A complex containing MUT-7 (fuchsia) and MUT-8/RDE-2 (light blue) mediates transition between the two steps in the RNAi process. At the most downstream step the RISC complex containing a single-stranded siRNA, the PAZ-PIWI protein, TSN-1 (dark burgundy) and VIG-1 (light green) are shown to target a mature mRNA. At the same time another complex containing a RdRP (either RRF-1 or EGO-1) (petrol blue above) and possibly RDE-3 (light brown) is engaged in the target-dependent amplification of the dsRNA. A similar complex containing RRF-3 (petrol blue below) is shown amplifying its target and creating competition to RdRP complexes involved in RNAi (Grishok, 2005).

3.2. *C. elegans* Nervous System

C. elegans nervous system is the most complex and best characterized tissue in the worm. An adult hermaphrodite nervous system has 302 neurons, belonging to two distinct and independent systems: a large somatic nervous system (282 neurons), and a small pharyngeal nervous system (20 neurons). *C. elegans* also has 56 neuronal support cells associated only with the somatic nervous system (White *et al.*, 1986). Communication between these neurons is facilitated by a vast network of 6400 chemical synapses, 900 gap junctions, and 1500 neuromuscular junctions (NMJs) (White *et al.*, 1986). Based on their topology and synaptic connection patterns, the neurons in the hermaphrodite have been categorized into 118 distinct classes. The majority of the neurons are monopolar, with a single unbranched process; only few neurons have more than two processes. The major synaptic regions in the animal are the circumpharyngeal nerve ring and the longitudinal ventral and dorsal nerve cords, which are connected via commissures. The nerve ring serves as a central hub for information exchange, receiving and sending signals to the rest of the body. Despite

its relative anatomical simplicity, the *C. elegans* nervous system contains many of the classical neurotransmitters found in vertebrates, including acetylcholine, glutamate, γ -aminobutyric acid (GABA), serotonin (5-HT), dopamine (DA), and neuropeptides (White *et al.*, 1986).

One of the main behaviors that allows *C. elegans* to survive is the locomotion. Underlying this aspect, 113 of the 302 *C. elegans* neurons are motoneurons (MNs) controlling “crawling” on solid surfaces and “swimming/trashing” in liquid media, as well as the motility of the alimentary and reproductive system. They belong to three different categories: A- and B-type MNs, which are cholinergic and stimulatory MNs; and D-type MNs (VD and DD MNs) which are GABAergic and inhibitory (White *et al.*, 1986; White *et al.*, 1976).

3.2.1. The GABAergic system of *C. elegans*

Inhibitory synapses using the neurotransmitter gamma-aminobutyric acid (GABA) are the most abundant in the vertebrate nervous system, and GABA acts in the central nervous system. γ -aminobutyric acid is an important neurotransmitter in *C. elegans* as well but, unlike in vertebrates it acts primarily at neuromuscular synapses. Specifically, GABA acts to relax the body muscles during locomotion and foraging and to contract the enteric muscles during defecation. Out of the 302 neurons, 26 produce the neurotransmitter GABA (Schuske *et al.*, 2004). These 26 GABA neurons are divided into different classes according to their synaptic outputs: 19 D-type neurons, including the 6 DD and 13 VD motoneurons (MNs), innervating the dorsal and ventral body muscles, respectively; 4 RME MNs which innervate the head muscles; the AVL and DVB MNs innervating the enteric muscles; and RIS, an interneuron (fig. 3.3).

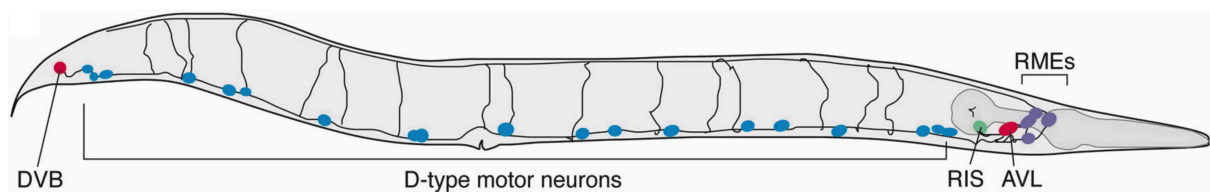


FIGURE 3.3. **The *C. elegans* GABA-ergic nervous system:** schematic drawing of the positions of the 26 GABAergic neurons. The VD and DD MNs innervate the body muscles (pale blue), the RME neurons innervate the head muscles (purple), the AVL and DVB neurons innervate the enteric muscles (red), RIS is an interneuron (green). Anterior is on the left and the left side of the body is shown (Schuske *et al.*, 2004).

The 19 D-types GABAergic motoneurons (GABA MNs) are inhibitory motoneurons and strictly postsynaptic to other MNs, from which they receive synaptic inputs. The dendritic arms of these neurons are on the opposite side to the axons. The DD neurons have short posterior process and a long anterior process emerging from the cell bodies. From the anterior process, a branch departs and runs laterally to form the commissure directed to the dorsal cord, which becomes an axon innervating the same region on that side.

The D-type MNs in *C. elegans* are involved in the control of the characteristic sinusoidal movement of the animal (figure 3.4A), which requires alternate contraction of the dorsal and ventral body wall muscles to propagate the body bends from one end to the other end of the body. Ventral cholinergic neurons release acetylcholine onto the body wall muscles of the ventral and dorsal side to excite

them and produce ventral and dorsal body bends, respectively. GABAergic neurons participate to body bends by releasing GABA and thus relaxing muscles on the opposite side of a contraction (figure 3.4B) (White *et al.*, 1976). DD MNs relax dorsal muscles, while VD MNs relax ventral muscles. D-type MNs are important for resetting the animal posture, for example to reverse movement direction or to initiate a rapid movement. Animals lacking D-type MNs and the relative GABA input for relaxing muscles, shrink in response to a touch, due to the hypercontraction of both dorsal and ventral muscles, induced by the presence of only excitatory input (fig. 3.4B). Animal can still adopt a sinusoidal posture, but the body waves amplitude is reduced.

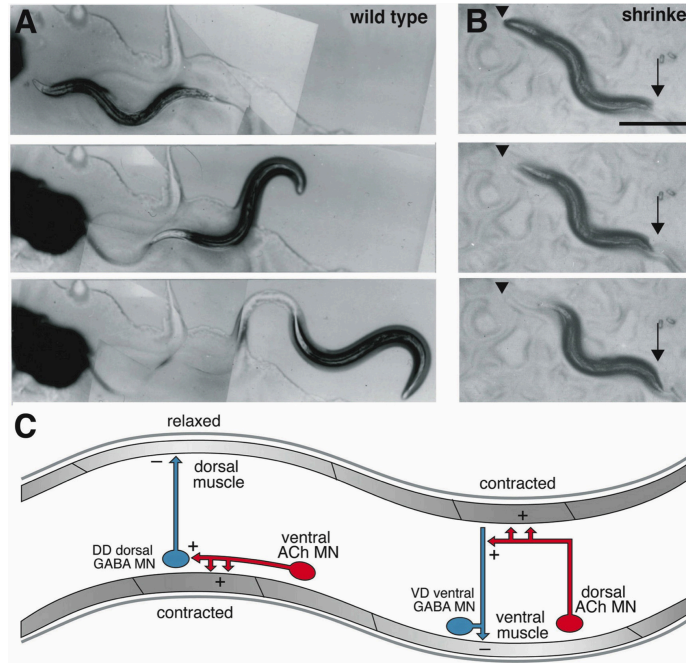


FIGURE 3.4. **The D-type motoneurons inhibit body wall muscles:** **A.** When a wild type worm is touched on the nose, it responds moving backward and performs a sinusoidal movement by propagating a wave from the tail to the head. **B.** When a mutant defective for GABA transmission is touched on the nose, it shrinks due to the contraction of muscles on both side of the body. **C.** MNs connectivity. DDs and VDs (blue) synapse to the dorsal and ventral body muscles respectively. Cholinergic MNs (red) send excitatory input to the ventral and dorsal muscles and to GABAergic MNs. Acetylcholine released by cholinergic MNs causes the contraction of the body wall muscles on one side of the worm and stimulates GABA release onto muscles on the opposite side. The stimulation and contralateral inhibition result in body bends and in coordinated locomotion. Anterior is to the left, ventral is down. (Schuske *et al.*, 2004).

3.3. *C. elegans* as model organism to study Spinal Muscular Atrophy

C. elegans, whose nervous system has been completely mapped and whose synaptic connections have been fully reconstructed (Cook *et al.*, 2019), has contributed to the understanding of several human neurodegenerative disorders. As a result, in recent years, many *C. elegans* models mimicking neurodegenerative disorders have been developed, including Spinal Muscular Atrophy (Roussos *et al.*, 2023).

3.3.1. *smn-1* gene in *C. elegans*

In *C. elegans* there is a protein that shows homology to human *SMN*. Like all animal models, worms have a single copy of *SMN* gene, *smn-1*. It was mapped on chromosome I and has 5 exons and 4 small introns. The full-length mRNA contains an open reading frame (ORF) of 624bp coding for

207 amino acids. The SMN-1 protein shows a significant overall homology (36%) to its human counterpart (figure 3.5), thus suggesting an associated functional role, conserved throughout evolution. The homology is significant at those regions considered functionally relevant: N-terminus, which in humans is involved in RNA binding and interaction with SMN interacting protein 1 (SIP1); the C-terminus, involved in SMN oligomerization; the Tudor domain, involved in the interaction with Smith core proteins. Moreover, also in *C. elegans*, the SMN protein maintains its ability to self-associate through the C-terminus and to interact with specific partners, such as SMI-1 and FIB-1, the *C. elegans* orthologs of human Gemin 2 and Fibrillarin, respectively (Miguel-Aliaga *et al.*, 1999).

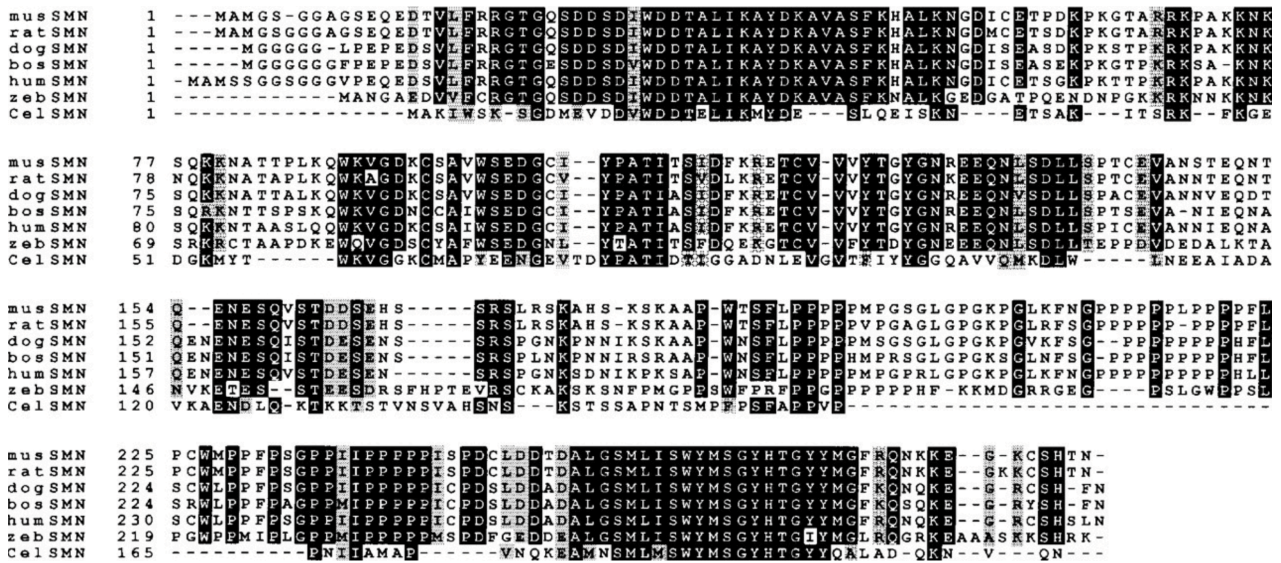


FIGURE 3.5. **Evolutionary conservation of SMN orthologs.** The overall similarity between the human and the nematode sequences is 36%. Homology is particularly significant at those regions that are thought to be functionally relevant. Species: *Mus musculus*; *Rattus norvegicus*; *Canis familiaris*; *Bos taurus*; *Homo sapiens*; *Danio rerio*; *Caenorhabditis elegans* (Miguel-Aliaga *et al.*, 1999).

C. elegans SMN-1 protein is expressed in all the nuclei of embryos from the zygotic stage. The presence of the SMN protein in such early stage is indicative that the protein and its mRNA are transmitted maternally during germline development. In the adult worm SMN-1 protein is ubiquitous and was found in most of the somatic cells, including neurons, body wall and vulvar muscles, hypodermal cells, gut and excretory cells (Miguel-Aliaga *et al.*, 1999).

Neurons and excretory cells of *C. elegans* are characterized by a strong cytoplasmic expression of SMN protein. By classical RNA interference, it was observed that decreased endogenous levels of SMN-1 protein have a dramatic effect on embryonic viability, while the few surviving animals show severe defects in neuronal, muscular and reproductive tissues with paralysis, vulvar abnormalities and sterility (Miguel-Aliaga *et al.*, 1999), thus confirming its essential role in the maturation and survival of oocytes and embryos.

3.3.2. SMA *C. elegans* models

To study the role of *smn-1* gene in *C. elegans*, two main genetic mutants have been generated through classical genetic approaches. The null mutant, *smn-1(ok355)*, characterized by the deletion

of most of *smn-1* gene (figure 3.6A), results in pleiotropic phenotypes, mainly represented by larval arrest and reduced lifespan (figure 3.6B) (Briese *et al.*, 2009). The few surviving larvae exhibit impaired motility (figure 3.6C), highlighting the crucial role of the SMN protein in neuromuscular function. However, no signs of neurodegeneration were observed (figure 3.6D). A possible explanation is that the null mutant is maintained as a balanced heterozygous, and consequently in the homozygous larvae the effects of *smn-1* loss in the nervous system could be masked due to the maternal contribution.

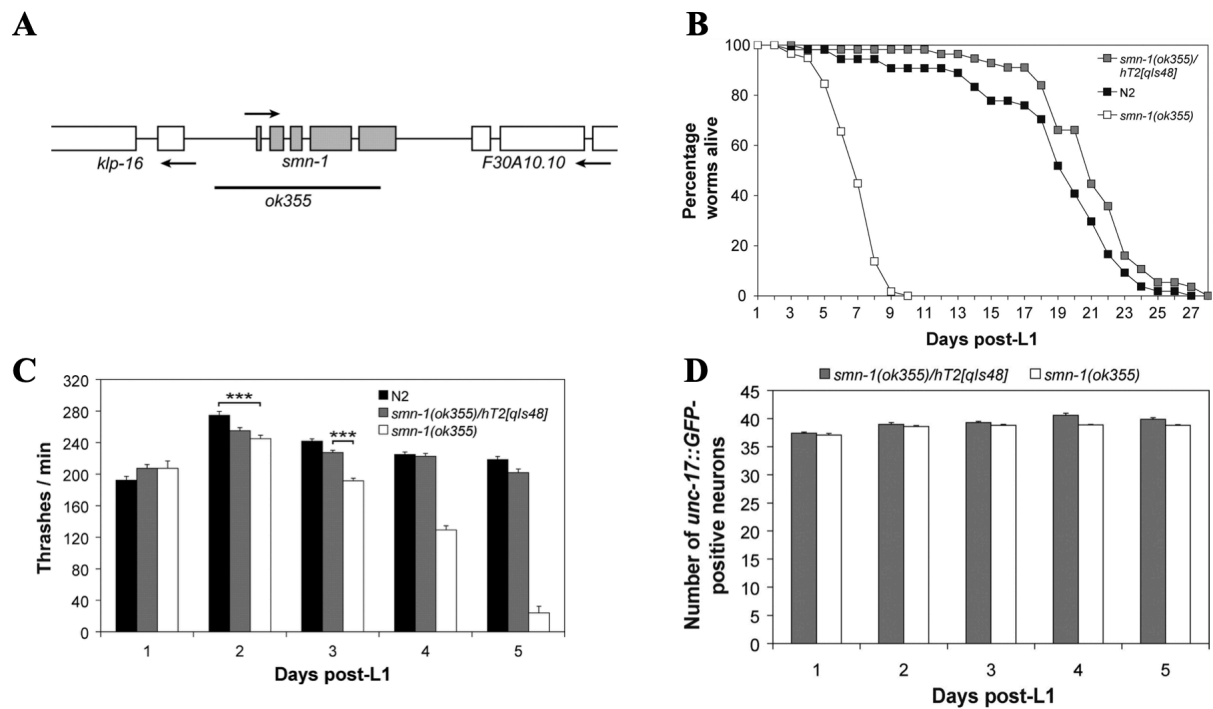


FIGURE 3.6. **The *smn-1(ok355)* mutant:** **A.** The *smn-1(ok355)* allele is considered a null allele due to a deletion which removes most of the gene, leaving only a part of exon 5. **B.** Survival of *smn-1(ok355)* homozygous worms, compared to wild type and *smn-1(ok355)* heterozygotes. Each data point represents the % of worms alive each specific day. **C.** Progression of the motility defect in *smn-1(ok355)* mutant (white bar), compared to wild type (black bar) and *smn-1(ok355)* heterozygous (gray bar) during development. **D.** Both *smn-1(ok355)* heterozygous and homozygous animals display no significant reduction in the number of neurons in the ventral cord, during development (adapted from Briese *et al.* 2009).

A second genetic mutant, *smn-1(cb131)*, expresses a hypomorphic allele that mimics a point mutation identified in a type III SMA patient (figure 3.7A). Despite their ability to reach adulthood, since their lifespan is not affected (figure 3.7B), these animals exhibit a defect in locomotion *in liquido* (figure. 3.7C), and lack any detectable changes in neuronal number or morphology (figure 3.7D), as well as body wall muscle morphology (Sleigh *et al.*, 2011). To explain this phenotype, synaptic functionality was tested and a defect was observed, suggesting that the defective locomotion in this model is due to the loss of the synaptic transmission between ventral nerve cord motor neurons and body wall muscles (Sleigh *et al.*, 2011).

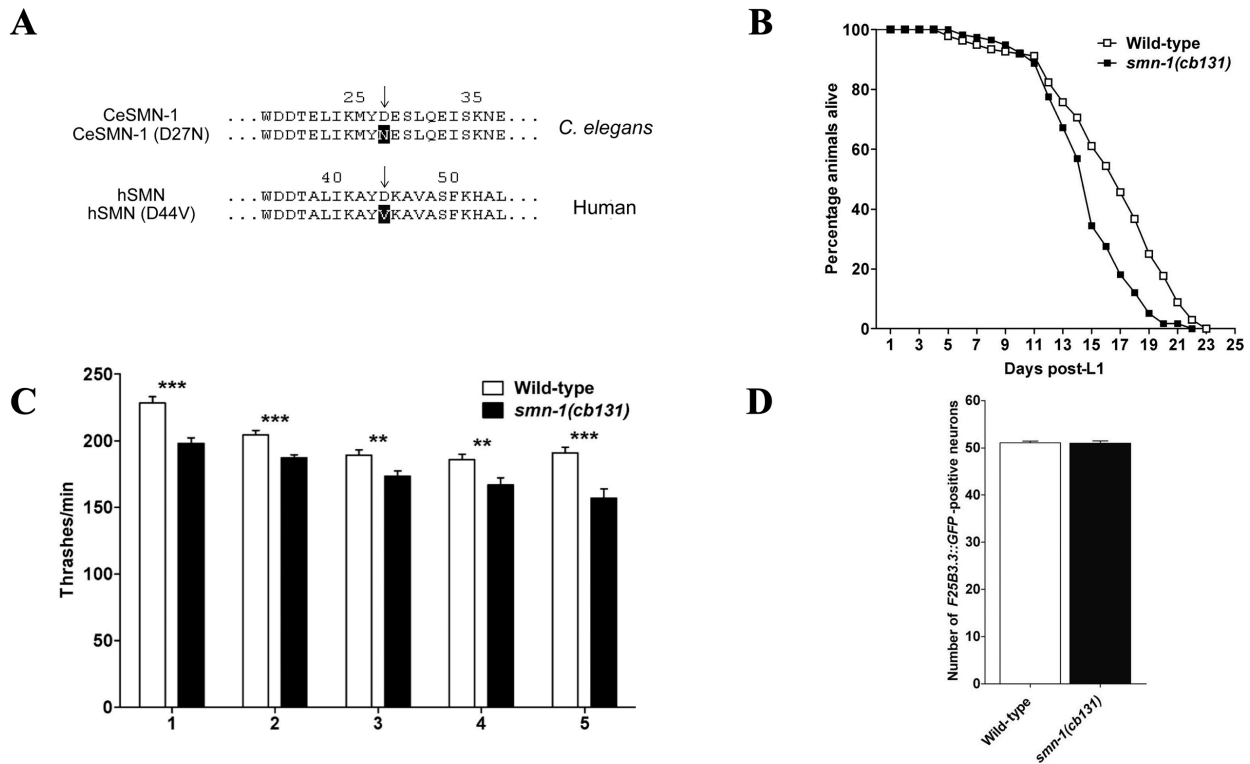


FIGURE 3.7. **The *smn-1(cb131)* mutant:** **A.** The *smn-1(cb131)* allele harbors a point mutation (D27N) which mimics a substitution found in a type III SMA patient (D44V). **B.** In *smn-1(cb131)* worms the lifespan is similar to that of wild type worms. **C.** *smn-1(cb131)* animals (black bar) show a defective motility in liquid medium compared to wild type animals (white bar). **D.** *smn-1(cb131)* mutant displays no reduction in the number of neurons in the ventral cord (adapted from Sleight *et al.* 2011).

The absence of specific signs of neurodegeneration in both genetic mutants limits the possibility of studying the SMA-associated neurodegenerative process *in vivo*. In this context a *C. elegans* model of *smn-1* loss-of-function was generated in 2016, using a neuron-specific RNAi strategy (Esposito *et al.*, 2007). This RNAi technique was able to efficiently reduce the function of *smn-1* gene in the 19 GABAergic motoneurons. The silencing method is transgene-driven and is based on the expression, under a GABAergic-specific promoter, of a sense and an antisense fragment corresponding to the gene of interest, *smn-1*. The resulting transgenic SMA model, namely *smn-1(MNs RNAi)*, is viable, healthy, fertile and does not exhibit the pleiotropic phenotypes observed in genetic models due to the systemic loss of *smn-1* function, but only some phenotypes specifically related to the *smn-1* knock-down in GABAergic motoneurons (Gallotta *et al.*, 2016). *smn-1(MNs RNAi)* animals display a defective backward movement (fig. 3.8A), a phenotype known to be dependent on GABAergic motoneurons function (Gallotta *et al.*, 2016). The loss of GABAergic motoneurons functionality, responsible for the defective backward movement, is caused by the loss of the neurons themselves through a degenerative process, which results in the disappearance of GFP-expressing GABAergic motoneurons, as assessed by using a GFP reporter (figure 3.8B).

The appearance of a degenerative process of GABAergic motoneurons triggered by *smn-1* silencing, makes this *C. elegans* model a suitable tool to study the SMA-related neurodegenerative process *in vivo*. It can help to understand why motoneurons are the cell type predominantly affected in patients, and which mechanisms contribute to neuronal cell death and disease progression.

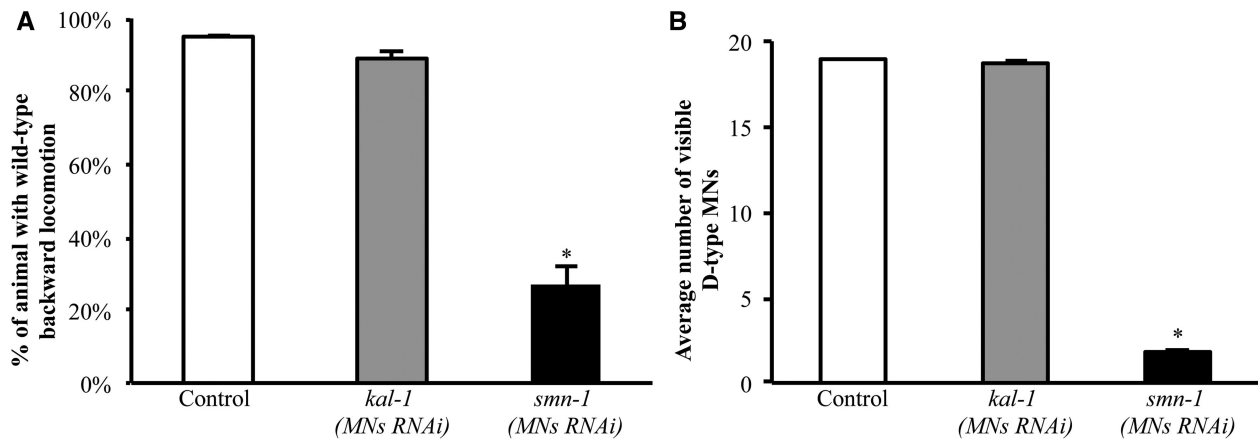


FIGURE 3.8. *smn-1* cell-specific knock-down causes defective backward locomotion and loss of D-type motor neurons: **A.** cell-specific knock-down of *smn-1* in D-type motor neurons results in alteration of motor neuron functionality. Each bar represents the mean percentage of animals with wild-type backward locomotion. *kal-1*(MNs RNAi) was used as negative control. **B.** average number of visible MNs after *smn-1*(MNs RNAi) knock-down and in negative controls (Gallotta *et al.*, 2016).

3.3.3. How *C. elegans* contributed in increasing knowledge in SMA field

Over the past decade, *C. elegans* SMA models have been extensively used to elucidate the molecular basis of Spinal Muscular Atrophy and identify novel therapeutic targets for SMA and molecules that could mitigate the deleterious effect of the disease.

Depletion of the cross-species SMN modifier *plst-1/PLS3* enhanced SMA-related phenotypes, confirming that *C. elegans* models of SMA can be successfully used to identify novel SMN modifiers (Gallotta *et al.*, 2016; Walsh *et al.*, 2020).

Several large-scale genetic screens have been carried out to identify genes involved in SMA, taking advantage of the small size and fast life cycle of *C. elegans*; in addition, automated microfluidic screening combined with custom image analysis software allowed the identification of 21 alleles that suppress MNs degeneration observed in *smn-1*(MNs RNAi) worms (De Carlos Cáceres *et al.*, 2018). Furthermore, the combination of -omics approaches and *C. elegans*, also, made a significant contribution in this field. Indeed, transcriptome analysis of pre-symptomatic *smn-1(ok355)* identified down-regulation of numerous nuclear-encoded mitochondrial genes and vacuolar H⁺-ATPase, and up-regulation of histone genes (Gao *et al.*, 2019).

Studies using *C. elegans* SMA models have highlighted the role of genetic SMA modifiers that, when depleted, suppress SMA-related phenotypes (Walsh *et al.*, 2020), as well as neuroprotective genes that when over-expressed are able to ameliorate the SMA phenotype, such as *tcab-1/WRAP53*, *hrp-2/SYNCRIP* and *tgs-1/TGS1* (Di Giorgio *et al.*, 2017; Rizzo *et al.*, 2019; Chen *et al.*, 2022).

C. elegans models of SMA have also been extensively used to identify neuroprotective compounds, including 4-aminopyridine (4-AP) (Sleigh *et al.*, 2011), which has been used in clinical trials for SMA type III patients (Chen, 2020); and natural extracts, such as, the green kiwi fruit able to rescue both the loss of visible MNs and the locomotion defect of the *smn-1*(MNs RNAi) model (Mazzarella *et al.*, 2019). Furthermore, studies using *C. elegans* SMA models confirmed the neuroprotective effect of drugs identified using SMA and other neurodegenerative models such as, Riluzole (Dimitriadi *et al.*, 2013; Doyle *et al.*, 2020), Valproic acid (VPA) (Gallotta *et al.*, 2016), Pimozide (Doyle *et al.*, 2020).

Caenorhabditis elegans is, therefore, a perfect complementary model organism to contribute to SMA field advancement.

3.4. *C. elegans* as complementary model organism to identify new SMA modifiers: *B-RAF/lin-45*

We recently employed *C. elegans* to investigate the role of *B-Raf* in SMA *in vivo*, as described in the work of Hensel *et al.* and in this thesis in Chapter 1. The *B-Raf* homolog in *C. elegans*, *lin-45*, belongs to the MAPK-ERK signaling pathway conserved in nematodes and it is expressed in both neurons and motor neurons, where it plays a role in locomotion (Coleman *et al.*, 2018).

As previously reported, B-RAF and phospho-B-RAF protein levels were significantly reduced in SMA mice compared to control animals. Therefore, to elucidate the potential involvement of *B-Raf/lin-45* in SMA pathway *in vivo* we conducted a rescue approach in the *smn-1*(MNs RNAi) model by over-expressing, through heat-inducible transgenics, a wild-type (*i-lin-45^{wt}*) and a hyperactive form of *lin-45* (*i-lin-45^{hyp}*). The latter is a S312A mutant that lacks an inhibitory phosphorylation site, leading to the protein hyper-activation (Kao *et al.*, 2004). The over-expression of the *lin-45^{hyp}*, but not of the wild-type form, partially rescued both the neurodegeneration and the defect in locomotion caused by *smn-1* deficiency, confirming also *in vivo*, a possible role of *B-RAF/lin-45* in SMA pathogenesis to be further characterized (figure 3.9) (Hensel *et al.*, 2021).

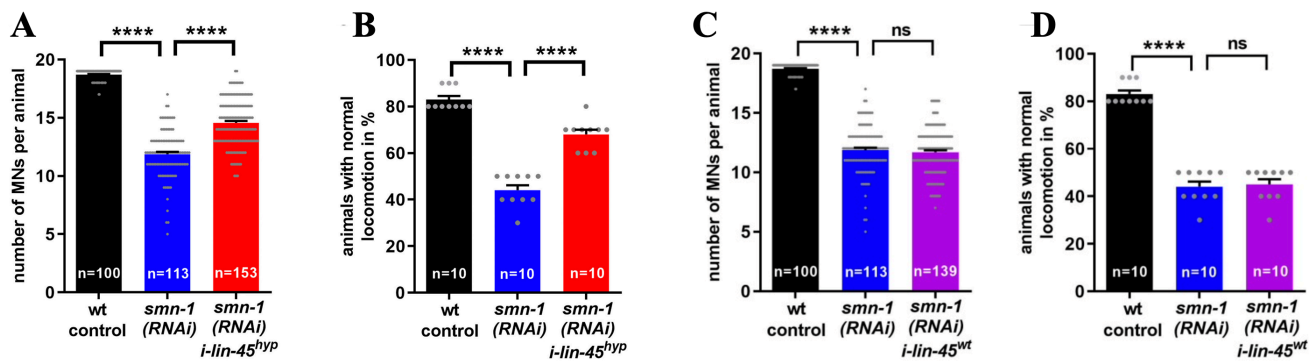


FIGURE 3.9. *i-lin-45* effect on motoneurons degeneration and locomotion defect in *smn-1* deficient *C. elegans*: A-B. *i-lin-45^{hyp}* is able to partially rescue both the loss of visible MNs and the defect in locomotion caused by the *smn-1* deficiency (red bars). C-D. *i-lin-45^{wt}* has no effect on both the loss of visible MNs and the defect in locomotion caused by the *smn-1* deficiency (purple bars). The black bars show the wild-type control animals, the blue bars show the *smn-1*(MNs RNAi) animals (Hensel *et al.*, 2021).

Progressive motoneurons degeneration is a hallmark of SMA which is recapitulated in *C. elegans* with the *smn-1* knockdown in motoneurons. The neurodegeneration starts from the first larval stage (L1) and increases with animal aging (Gallotta *et al.*, 2016). Taking advantage of this aspect, we tested whether the degeneration can be rescued by expressing *i-lin-45^{hyp}* at later stages. Using the heat-inducible promoter, *lin-45* was expressed starting from different larval stages and its kinetics in motoneuron degeneration was characterized. The most efficient rescue was obtained with a *lin-45^{hyp}* expression started from the embryonic stage. However, an induction at L1 and even L2 larval stages was sufficient to substantially prevent motoneurons degeneration (figure 3.10). This is interesting

since at L2 stage all 19 D-types motoneurons have already been generated, thus demonstrating that *lin-45* protects from motoneuron loss rather than interfering with neurogenesis. Moreover, motoneuron degeneration starts at L1 in this model and *lin-45* is able to rescue the degeneration even after the onset of motoneuron loss (Hensel *et al.*, 2021).

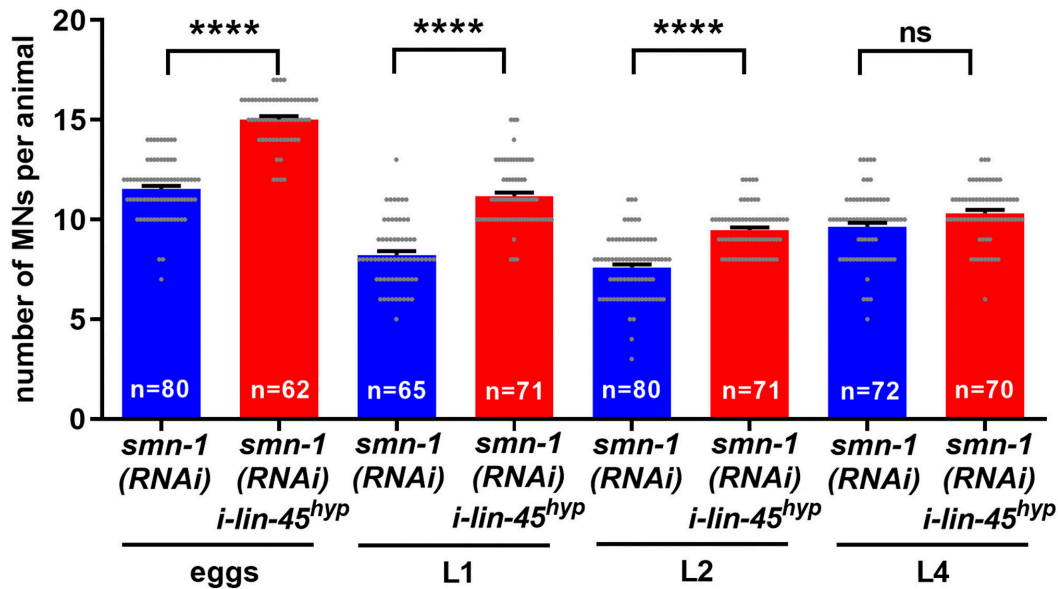


FIGURE 3.10. *i-lin-45^{hyp}* effect on motoneurons degeneration after temperature mediated induction: the picture shows how *i-lin-45^{hyp}* expression is able to partially rescue the neurodegeneration caused by *smn-1* deficiency when started in embryos and at early larval stages (L1-L2) (red bars), when the neurogenesis is completed and the neurodegeneration already started. Blue bars show the *smn-1*(MNsRNAi) model (Hensel *et al.*, 2021).

These data, obtained during my MSc thesis project, revealed the neuroprotective effect of *B-Raf/lin-45* in SMA. They were the first steps towards a characterization of the network of dysregulated proteins identified by Prof. Claus group. All these results together with the preliminary data obtained by Prof. Claus group (see Chapter 1 paragraph 1.5) were published in the paper by Hensel *et al.*, 2021.

In this PhD project, I further investigated the role of B-Raf/*lin-45* in neurodegeneration and the results obtained are described in Chapter 7, these results were also published in Hensel *et al.*, 2021. Then I studied the remaining 37 proteins of the network (Chapters 8 to 10) and validated my results in mammalian models (Chapter 12).

Materials and Methods

CHAPTER 4

Caenorhabditis elegans methods

4.1. Materials

Chemicals were obtained from Sigma-Aldrich. The extracts from *Vigna unguiculata* (Cowpea Seed) were kindly provided by Prof. P. Coccetti (Università Milano Bicocca, Dipartimento di Biotecnologie e Bioscienze, Milan). MEK (U0126, Calbiochem®) and ERK (FR180204, Calbiochem®) inhibitors were kindly provided by prof. dr. P. Claus (SMATHERIA gGmbH – Non-Profit Biomedical Research Institute, Hannover). HT115 RNAi bacteria carrying constructs for gene silencing, and the empty vector were obtained from the Ahringer RNAi library supplied by Source BioScience Ltd. and sequence verified. The PureLink Quick Plasmid MiniPrep Kit (Invitrogen) was used to purify the constructs of interest prior to sequencing.

4.2. Cultivation

Caenorhabditis elegans strains were grown under uncrowded standard condition, at 20°C or 25°C on solid or liquid medium. Solid medium was made of Nematode Growth Medium (NGM) agar plates seeded with *Escherichia coli* strain OP50, as described by Brenner (Brenner, 1974); liquid medium was made of M9 1× buffer and cholesterol medium using dead *E. coli* strain OP50 as food source.

4.2.1. Growth *in solido*

Nematodes were grown and handled according to standard procedures under uncrowded conditions on Nematode Growth Medium (NGM) agar plates seeded with *Escherichia coli* strain OP50 (Brenner, 1974). All strains were maintained at 20°C unless otherwise stated.

NGM is a chemically defined medium used to cultivate and maintain nematode strains. One liter of solution is made of: 2.5 g of peptone; 3g of sodium chloride; 20g of agar and 972 mL of distilled water. The solution is sterilized in an autoclave before use.

The protocol for preparing plates for growing animals on solid medium consists in preparing NGM as described. After autoclaving, while working under a laminar flow hood, the following components need to be added to the media: 1mL of MgSO₄ 1M, 1 mL of CaCl₂ 1M, 1mL of cholesterol in ethanol and 25mL of NaKPO₄ (pH 6.4). Finally the NGM is poured in plates. After the plates are seeded with *Escherichia coli* strain OP50 grown overnight in liquid LB medium at 37°C with agitation.

4.2.2. Growth *in liquido*

For drug screening in very small volumes and on a high number of animals, it is often useful to grow animals from eggs to adulthood *in liquido*.

Nematode Liquid Growth Media (NLGM) consists of M9 1× buffer (3 g KH₂PO₄, 6 g Na₂HPO₄, 5 g NaCl, 1 mL MgSO₄ 1M, H₂O to 1 L) sterilized by autoclaving and supplemented with cholesterol 5mg/ml and dead *E. coli* strain OP50 as food source.

The protocol for preparing plates for growing animals in liquid medium consists in centrifuging 15 mL of OP50 5' at 3,000 g in 50 mL tube. The obtained pellet is first resuspended in few mL of M9 1× buffer with a glass pipette, then M9 1× buffer is added to reach the final volume of 50 mL. The solution is then vortexed for 1h to ensure complete resuspension of the bacterial pellet. Finally, 1 mL of antibiotic/antimycotic solution (100× concentration) is added to achieve the final concentration of 2×. Additionally, 50 µL of Cholesterol (5mg/mL stock solution) is added to reach a final concentration of 5ng/mL in the complete M9 1×-OP50 solution.

In this work, the growth *in liquido* was performed in a 96-well plate, so 1-2 mL of NLGM containing eggs pellet have been collected (see paragraph 4.2.3), and the egg pellet was diluted in order to obtain ~40 eggs each 60 µL. Then the plate was transferred in an incubator at 20°C on a swinging base for 5 days.

4.2.3. Synchronization of worms

Sometimes it is essential to synchronize *C. elegans* to the same larval stage. A synchronized population of worms can be useful for a wide range of experiments that don't allow for age differences such as genetic screening, drug testing or behavioral assays. The synchronization protocol that allows a large number of eggs to be obtained in short time is by bleaching. It consists of treating adult worms with a bleaching solution to collect eggs, that are moderately resistant to bleaching. The collected eggs are then transferred to a solid or liquid growth medium to generate a new synchronous population.

Bleaching protocol consists of several steps:

1. Starting from plates with many gravid adult hermaphrodites and eggs, collect them in a Falcon tube by washing the plates with M9 1× buffer or distilled water
2. Centrifuge at 1,300 g for 1' and remove the flow-through by using a vacuum pump
3. Preparing the bleaching mix by adding 500 µL of NaOH 5M (final concentration 0.5M), 385 µL of Sodium hypochlorite 13% (final concentration 1%) (SIGMA)
4. Add 4,115 µL of M9 1× buffer and 885 µL of bleaching mixture to the worm pellet. Seal the tube with parafilm to avoid leaks
5. Shake by hand vigorously and vortex, check after 10' under a dissecting microscope for any eggs released into the bleach solution. If worm cadavers are still present, vortex continuously for 2' to 5', checking frequently under the dissecting microscope, until all cadavers have disappeared
6. Add M9 1× buffer up to 15 mL to block the reaction and centrifuge at 1,300 g for 3'
7. After the centrifugation a wispy pellet will be visible, remove the flow-through by using a vacuum pump, and wash by adding 15 mL of M9 1× buffer. Centrifuge 1,300 g for 3'. After this centrifugation the pellet will be clean and eggs clear
8. Perform other 3 washes with M9 1× buffer to remove all bleaching solution.

9. Transfer the eggs in a new NGM plate or add 1-2 mL of NLGM to each tube containing eggs pellet.

4.3. Strains

The strains used in this work were provided by: the *Caenorhabditis* Genetic Center (CGC) funded by NIH Office of Research Infrastructure Programs (P40OD010440); the *C. elegans* research community groups. Particularly, the strain IK105 was provided by I. Mori's group and LL2073 was provided by Anne Hart's group provided us. Other strains were either previously obtained in Di Schiavi's group or generated for this study through genetic crosses or transgenesis. All strains and their sources are listed in Table 4.1.

4.3.1. Genetic crosses

Genetic crosses were made to transfer transgenes to the appropriate genetic background. A genetic cross in *C. elegans* is usually carried out by using non-starved young adult animals (1 day post L4). Hermaphrodites and males are transferred in a 3:1 ratio to a new plate with a minimal surface area covered with bacteria to force physical proximity and thus increase the mating. A paternal visible marker was used to select the "non-self" F1 progeny from "self" progeny, that was moved to a fresh plate to identify our strain of interest. In case of a genetic mutant allele, its presence was verified by polymerase chain reaction (PCR) genotyping (see Table 4.2 for prime list). At least two independent isogenic clones were examined after each crossing. All strains were kept at 20°C unless otherwise stated. In order to express *csIs34* heat-inducible transgene, a continuative heat-treatment maintaining animals at 25°C has been performed.

4.3.2. Transgenesis

The construct GBF377 (*punc-47::lin-45^{wt}*) for MNs-*lin-45^{wt}* expression was created by two-step PCR fusion (Hobert, 2002) of the *unc-47* promoter with *lin-45* gene, both amplified from the genome of wild type strains. The transgenic construct GBF378 (*punc-47::lin-45^{hyp}*) for MN-*lin-45^{hyp}* expression was similarly created by PCR fusion of *unc-47* promoter with *lin-45^{S312A}* mutated gene. This time a three step PCR fusion approach was used: in the first stage the 5'-end and the 3'-end of *lin-45* gene were separately amplified from wild type genomic sequences, using adequate overlapping primers carrying a T>G substitution in the position +2578 of the *lin-45* gene (isoform *c.1*); then we created the *lin-45^{S312A}* mutated version of the gene by fusing these two fragments with nested primers and the nucleotide change was confirmed by Sanger sequencing; finally, in the third stage, the amplicon with the promoter and the amplicon with the mutated gene were fused as above using nested primers (see Table 4.3 for prime list). Germline transformation was accomplished as described (Mello *et al.*, 1991), by injecting a DNA mixture containing the constructs at 2 ng/μL (lower concentration, LC) and 20 ng/μL (higher concentration, HC) into the gonads of NA1721 [*juIs76, gbIs4*] (Gallotta *et al.*, 2016) adult animals together with a phenotypic marker for the selection of the transgenic progeny. For this purpose we used plasmid pJM371

[*pelt-2::RFP*], a kind gift of Prof. J.D. McGhee (University of Calgary), as co-injection marker at 30 ng/μL. This plasmid allows to recognize transgenic animals through RFP expression in the nuclei of intestinal cells (McGhee *et al.*, 2008). At least three independent transgenic lines were examined for each concentration. Genetic crosses were performed to obtain strains without *juIs76* for locomotion assays.

Strain Number	Genotype	Use	Source
N2 (Bristol variety)	wild-type	qPCR and Behavior assays	CGC
LM99	<i>smn-1(ok355) I/hT2 [bli-4(e937) qIs48] (I;III)</i>	qPCR and Behavior assays	CGC
MT8666	<i>mek-2(n1989) I</i>	Genetic Cross	CGC
HC114	<i>cclIs4251 [pmyo-3::GFP::LacZ::NLS + pmyo-3::mitochondrial GFP + dpy-20(+)] I; qtlIs3 [myo-2p::GFP dsRNA hairpin] III; mIs11 [myo-2p::GFP + pes-10p::GFP + gut-promoter::GFP] IV; sid-1(qt9) V</i>	Genetic Cross	CGC
9 words omitted			
KP3948	<i>eri-1(mg366) IV;lin-15(n744) X</i>	Genetic Cross	CGC
NA1064	<i>gbEx211 [punc-25::GFPRNAisas; pchs2::dsRed2]; oxIs12 [unc-47p::GFP + lin-15(+)] X</i>	Genetic Cross	EDS lab
LL2073	<i>smn-1(cb131) I</i>	Genetic Crosses Behavior assays RNAi by feeding	Anne Hart's group
NA1618	<i>gbEx593b [GBF109 punc-25::smn-1 RNAi sas; pchs-2::gfp]</i>	Genetic Cross	EDS lab
HA2031	<i>rtIs31 [pelt-2::GFP + posm-10::HtnQ150] X</i>	Genetic Cross	CGC
12 words omitted			
NA1330	<i>gbIs4[GBF109 punc-25::smn-1(RNAi), GB301 pchs-2::GFP] III</i>	Genetic Cross, Behavior assay, qPCR	EDS lab
NA1335	<i>gbIs4 III;oxIs12 X</i>	Drug treatment and Phenotypic analysis	EDS lab
NA1719	<i>juIs76 [punc- 25::GFP, lin-15(+)] II</i>	Phenotypic analysis	EDS lab
NA1721	<i>gbIs4III; juIs76 II</i>	Phenotypic analysis	EDS lab
NA2082	<i>wplIs36 [punc-47::mCherry] I; wpSi1 [punc-47::rde-1::SL2::sid-1 + Crb-unc-119] I; gbls4 III; eri-1(mg366) IV;lin-15(n744) X</i>	Genetic Cross	EDS lab
NA2084	<i>juIs76 II; gbls4 III; csIs34[phsp-16.41::lin-45^{S312A}, unc-119(+)] X</i>	Genetic Cross and Phenotypic analysis	Genetic Cross
XE1375	<i>wplIs36 [punc-47::mCherry] I; wpSi1 [punc-47::rde-1::SL2::sid-1 + Crb-unc-119] I; eri-1(mg366) IV; rde-1(ne219); lin-15(n744) X</i>	RNAi by feeding screening and Phenotypic analysis	CGC
NA2107	<i>wplIs36 [punc-47::mCherry] I; wpSi1 [punc-47::rde-1::SL2::sid-1 + Crb-unc-119] I; gbls4 III; eri-1(mg366) IV; rde-1(ne219) V; lin-15(n744) X</i>	RNAi by feeding screening and Phenotypic analysis	EDS lab

NA2108	<i>juls76 II; gbls4 III; csIs42[phsp-16.41::lin-45^{wt}, unc-119(+)]</i>	qPCR	Genetic Cross
NA2269	<i>juls76 II; gbls4 III; gbEx679a-c [GBF377 punc-47::lin-45^{wt}LC, pJM371 pelt-2::RFP]</i>	Genetic Cross and Phenotypic analysis	Trangenesi
NA2270	<i>juls76 II; gbls4 III; gbEx678a-c [GBF377 punc-47::lin-45^{wt}HC, pJM371 pelt-2::RFP]</i>	Genetic Cross and Phenotypic analysis	Trangenesi
NA2271	<i>juls76 II; gbls4 III; gbEx682a-c [GBF377 punc-47::lin-45^{hyp}LC, pJM371 pelt-2::RFP]</i>	Genetic Cross and Phenotypic analysis	Trangenesi
NA2272	<i>juls76 II; gbls4 III; jgbEx683a-b [GBF377 punc-47::lin-45^{hyp}HC, pJM371 pelt-2::RFP]</i>	Genetic Cross and Phenotypic analysis	Trangenesi
NA2273	<i>gbls4 III; gbEx679c</i>	Behavior assays	Genetic Cross
NA2274	<i>gbls4 III; gbEx682a</i>	Behavior assays	Genetic Cross
NA2275	<i>gbls4 III; gbEx683a</i>	Behavior assays	Genetic Cross
NA2276	<i>gbls4 III; gbEx678b</i>	Behavior assays	Genetic Cross
NA2277	<i>mek-2(n1989) I; juls76 II; gbls4 III; csIs34 X</i>	Drug treatment and Phenotypic analysis	Genetic Cross
NA2294	<i>rtIs31 X; gbEx211</i>	Phenotypic analysis	Genetic Cross
NA2297	<i>wpls36 I; gbls4 III; eri-1(mg366) IV; lin-15(n744) X</i>	RNAi by feeding screening and Phenotypic analysis	Genetic Cross
NA2300	<i>wpls36 I; gbls4 III</i>	RNAi by feeding screening and Phenotypic analysis	Genetic Cross
NA2304	<i>wpls36 I</i>	RNAi by feeding screening and Phenotypic analysis	Genetic Cross
NA2305	<i>ccls4251 I; gbEx211</i>	Phenotypic analysis	Genetic Cross
NA2323	<i>ccls4251 I; sid-1(qt9) V; gbEx211</i>	Phenotypic analysis	Genetic Cross
NA2324	<i>ccls4251 I; sid-1(qt9) V</i>	Phenotypic analysis	Genetic Cross
NA2325	<i>wpls36 I; eri-1(mg366) IV; lin-15(n744) X; gbEx593b</i>	Phenotypic analysis	Genetic Cross
NA2330	<i>sid-1(qt9) V</i>	-	Genetic Cross
NA2331	<i>wpls36 I; eri-1(mg366) IV; lin-15(n744) X</i>	RNAi by feeding screening and Phenotypic analysis	Genetic Cross
10 words omitted			
10 words omitted			
12 words omitted			
12 words omitted			

10 words omitted			
10 words omitted			
NA2399	<i>smn-1(cb131) I;eri-1(mg366) IV;lin-15(n744) X</i>	-	Genetic Cross

TABLE 4.1. *C. elegans* strains used: the table above shows the strain used/obtained for the whole work and their genotypes (first and second column); the third column shows for which purpose each strain was used (drug test, RNAi by feeding, genetic crosses, qPCR, phenotypic analysis or behavioral assay); the fourth column shows each strain source.

4.4. Drug treatments

Compounds 1, 2 and 3 (Sigma Aldrich) were tested *in liquido* as previously described. They were dissolved in dimethylsulfoxide (DMSO, Sigma Aldrich) and then added to NLGM to reach a final concentration of 100 μ M in DMSO 1%. DMSO 1% was used as mock. Neurodegeneration was scored at young adult stage (1 day post-L4). Each experimental condition was run blindly in triplicate.

Vigna unguiculata (Cowpea Seed) extract, supplied as water-soluble powder, was tested *in solido* at two different final concentrations, 0.2% w/v and 0.5% w/v. First, two stock solutions, 2% w/v and 5% w/v, were prepared by dissolving the extract in H₂Odd. Each solution was then sterilized by a sequential filtration with 0.44- μ m and 0.22- μ m membrane filters (Millipore). Then, to obtain the desired final concentration, a specific volume of the extract was added on top of the plates containing a given volume of NGM agar previously poured and cooled down to avoid exposing the extract to high temperatures of molten agar and, thus, preserving the chemical stability of the natural extract components. Plates were allowed to dry for 1 day before being seeded with heat-killed OP50 bacteria to avoid any contribution of the bacterial metabolism to the assay. L4 worms were then transferred onto the extract/control plates and left for 24 hours to reach the adult stage and then to lay eggs, before being removed. Animals among the progeny were scored for neurodegeneration phenotype at young adult stage (1 day post L4). H₂Odd was used as mock, and 3mM Valproic acid as positive control. Each experimental condition was run in triplicate.

For treatments with MEK (U0126, Calbiochem®) and ERK (FR180204, Calbiochem®) inhibitors, *i-lin-45^{hyp};smn-1(RNAi)*, wild type and *smn-1(RNAi)* animals have been grown at 25°C, control strains at 20°C *in solido*. Animals were synchronized by bleaching and when they reached L2 stage (after ~28h at 20°C or ~20h at 25°C), 40 animals have been transferred on plates seeded with dead OP50 bacteria and 20 μ M of each inhibitor in DMSO 1%. DMSO 1% was used as mock. Neurodegeneration has been scored at young adult stage (1 day post-L4) and treatments were performed in triplicates.

4.5. RNAi by feeding

RNAi experiments were performed by optimizing Kamath *et al.*, 2000 protocol. dsRNA for silencing were obtained from Ahringer RNAi library (Table 4.2 omitted). The procedure consists of three steps: bacterial growth, plates preparation and worms treatment.

Bacteria growth: colonies of HT115 bacteria containing RNAi plasmids, empty for control

(L4440) or carrying the dsRNA of interest, were picked from the Ahringer RNAi library at -80°C and grown overnight at 37°C on LB agar plates supplemented with ampicillin (final concentration 0.05 mg/mL) and tetracycline (final concentration 0.005 mg/mL) to select the colonies carrying the RNAi construct. Single colonies were, then, picked and grown again over-night at 37°C on LB agar plates. The next day, single colonies were picked and grown in culture in LB with ampicillin (final concentration 0.05 mg/mL).

Plates Preparation: NGM was prepared as previously described and supplemented with IPTG (final concentration 1mM) and ampicillin (final concentration 0.05 mg/mL). Plates were dried overnight, seeded with HT115 bacteria and stored at 4°C. To induce the dsRNA expression, the seeded plates were incubated overnight at 20°C.

Worm treatment: After induction of dsRNA expression, a maximum of 10 L4 animals were placed on IPTG plates, according to the strain used. After two generations, the desired phenotypes (loss of visible motoneurons, thrashing and lifespan) were analyzed. In all RNAi experiments, animals were simultaneously examined in triplicates and *mom-2* silencing has been used in parallel as positive control for the RNAi approach, since its silencing causes lethality.

Table 4.2 omitted

TABLE 4.2. **dsRNA used in the RNAi by feeding screening:** the table above shows the names of the *C. elegans* candidate genes silenced (columns A, C and E) and their location in the Ahringer RNAi library (columns B, D and F) .

4.6. Phenotypic assays

The three *C. elegans* SMA models tested in this work exhibit several SMA-related phenotypes, as previously described (see Chapter 3 paragraph 3.3.2). In this work, I analyzed: neurodegeneration and defects in backward locomotion caused by *smn-1* silencing, *smn-1(MNs RNAi)*; the defect in thrashing locomotion characterizing the *smn-1(cb131)* model; and the lifespan defect shown by the severe SMA model, *smn-1(ok355)*. Furthermore, I also investigated the putative spreading effect of the RNAi mediated by transgenes in neurons by testing GFP-silencing in intestine and muscles.

4.6.1. Microscopy analysis

For microscopy analysis young adult animals (1 day post L4) were immobilized with 0.01% tetramisole hydrochloride (Sigma-Aldrich) on 4% agar pads. The degenerative phenotype (referred as neurodegeneration in the following sections) was scored counting the number of visible D-type motoneurons expressing GFP per animal, using a motoneuron specific promoter (*punc-25::GFP*) and a Zeiss Axioskop microscope equipped with epifluorescence and DIC Nomarski optics with a 40x objective. The number of animals analyzed each time (n) has been added to graphs. Confocal images were collected with Leica TCS SP8 AOBS microscope, using 20× objective.

To evaluate the spreading effect in intestine and muscle, worms have been analyzed using a fluorescence-equipped stereo microscope Leica M205FCA, using 2× objective. For each data set I have evaluated the percentage of animal expressing GFP in which the green signal was completely present, partially present or absent in the tissue of interest. The number of animals analyzed (n) has been added to graphs. Images were collected with Leica DM600 microscope, using 20× objective.

4.6.2. Locomotion assays

To measure motility and motor function, animals were tested for backward locomotion or their ability to move in liquid.

To test GABAergic motoneuron functionality the backward movement locomotion assay was performed blindly, on NGM agar plates, 3 cm in diameter, seeded with *E. coli* strain OP50. Using an eyelash, the animals were gently touched first on the tail to induce a forward movement and then on the head to induce a backward movement. A defect in backward movement was scored when animals were unable to fully move back. Locomotion was scored for increments of ten animals per replicate and the percentage of normal locomotion was calculated, with a total of 100 animals scored.

To assess locomotor performance following RNAi silencing of genes of interest, the thrashing assay was performed on synchronized animals 1-days post L4. Animals were grown on RNAi *by feeding* plates for two generations as previously described. To obtain synchronized worms, F1 adults laid eggs for 2h on new RNAi plates. F2 synchronized animals were then transferred to a new RNAi *by feeding* plate the day before analysis to avoid starvation. Shortly before the analysis, they were moved to a blank plate to remove bacteria, then placed in 5 μ L sterile distilled water in a sterile watch glass, allowed to acclimate for 5 min and then video recorded for 30". The number of thrashes per minute was assessed by counting each change of direction with respect to the longitudinal axis of the body. Videos were recorded using Leica LAS EZ software. A minimum of 90 animals per condition were analyzed.

4.6.3. Lifespan assay

To evaluate the lifespan of the knock-out animals following RNAi silencing of genes of interest, worms were grown on RNAi *by feeding* plates for two generations (see paragraph 4.5). Two days after the hatching, F2 L2 homozygous *smn-1(ok355)* worms were selected and the survival test started. Survival was assessed, by slightly tapping them with an eyelash daily for live animals, dead animals and censored animals. If animals did not respond, they were considered dead, scored as such and removed. Life span assay wasn't performed blindly.

4.7. Molecular Biology

4.7.1. DNA isolation

Nematode genomic DNA was extracted using the following protocols.

GENOMIC DNA EXTRACTION FROM A POPULATION:

1. Wash worms and harvest them from NGM agar plates
2. Centrifuge the samples for 5' at 3,000 rpm and discard the supernatant
3. Add to sample a Lysis buffer (0.1 M TrisCl pH 8.5, 0.1 M NaCl, 50 mM EDTA pH 8.0, 1% SDS) with 20 mg/mL of Proteinase K and incubate at 60°C for 1 hr. Proteinase K was then inactivated by incubation for 20' at 95°C. 1 μ L of the lysates was used for PCR reactions.

GENOMIC DNA EXTRACTION FROM A SINGLE WORM:

1. Prepare the Lysis solution: Lysis buffer (0.1 M TrisCl pH 8.5, 0.1 M NaCl, 50 mM EDTA pH 8.0, 1% SDS) with 20 mg/ml of Proteinase K
2. Aliquot 6 μ L of Lysis solution into PCR tubes
3. Put one gravid worm in every tube-
4. Incubate samples at 60°C for 1h. Proteinase K was then inactivated by incubation for 20' at 95°C. 1 μ L of the lysates was used for PCR reactions.

4.7.2. Polymerase Chain Reaction (PCR) for genotyping

Strains obtained by genetic crosses were genotyped by PCR using Fast Start Taq Polymerase (Roche). The reaction mixture for a single sample is the following:

- 15.8 μ L sterile ddH₂O
- 0.5 μ L dNTP 10 mM
- 2.5 μ L Buffer Fast Start 10 $\times$
- 2.5 μ L Primer Forward 4 μ M (see Table 4.2)
- 2.5 μ L Primer Reverse 4 μ M (see Table 4.2)
- 0.2 μ L Taq Fast Start (Roche)
- 1 μ L DNA

4.7.3. RNA isolation and quantitative real-time PCR

500 L2 animals (wild type, *smn-1 KO*) or pellets of young adult animals (1 day post-L4) (wild type, *smn-1(RNAi)*, *smn-1(RNAi); i.lin-45^{wt}*, *smn-1(RNAi); i.lin-45^{hyp}*) grown at 15° and 25°C were flash-frozen three times using dry-ice/ethanol and thawing at 37°C, before proceeding with RNA extraction in TRI Reagent® (Merck), according to the TRI Reagent® protocol. RNA was treated with DNaseI-RNase-free (Thermo Scientific). In transgenic lines *csIs34* and *csIs42* (*i.lin-45^{wt}* and *i.lin-45^{hyp}*), *lin-45* has been cloned as cDNA, hence mRNA is indistinguishable from gDNA. Therefore, to ensure that gDNA was completely eliminated from transgenic strains, 1 μ g of RNA was incubated with 2U of DNase for 2h at 37°C. Then complete gDNA removal was verified by PCR analysis using specific oligonucleotides designed to amplify the promoter region of the transgene. Reverse transcription was performed with SuperScript Reverse Transcriptases (Invitrogen) and the steady-state mRNA abundance was determined using the Power SYBR Green PCR Master Mix (Applied Biosystems) on the 7900HT Fast Real Time PCR System (Applied Biosystems), following standard procedures. Expression levels were normalized to the average level of the housekeeping gene *act-1*. Each experiment assay was performed in triplicate in three independent experiments. The oligonucleotide sequences for transcript analysis are listed in Table 4.3.

Application	Primer name	Sequence 5'⇒3'
<i>lin-45</i> expression level by real-time PCR	<i>ActRTfw</i>	CCCAATCCAAGAGAGGTATCCT
	<i>ActRTrev</i>	ATATCATCCCAGTTGGTGACGAT
	Fw <i>RT lin-45</i>	GATGCACAACGACCCAGGCC
	Rev <i>RT lin-45</i>	CGGTACCGAAGGATCCCGAACC
	Fw <i>hsp-16.41</i>	CCAACACGCTTTGTTCTAGTGC
	Rev <i>lin-45fgDNA g</i>	GAGGAGTTGAAGGACTTGCC
MN specific expression of <i>lin-45^{wt}</i> and <i>lin-45^{hyp}</i>	Fw <i>punc-47Ext</i>	CTGTCAAAGGGGTACAGCATG
	Rev <i>punc-47</i>	CTGTAATGAAATAAATGTGACG
	Fw <i>punc-47Lin-45</i>	GCGTCACATTTATTTTCATTACAGATGTCTGCAATG GCTGTACTG
	Rev <i>Exinj lin-45</i>	CAAATACTGTGGTGCAGCTTC
	Rev <i>oMS104</i>	GATATTCGGAGCAGCAGAGCTCCGATC
	Fw <i>oMS103</i>	GATCGGAGCTCTGCTGCTCCGAATATC
	Rev <i>lin-45 Ext2</i>	CCATTCGTCCGACATCCTGTGG
	Fw <i>punc-47 Int</i>	CCCGGAACAGTCGAAAGTCG
	rev <i>Inj lin-45</i>	GAAGAAACAGGAAGTCAGGCG
	Rev <i>lin-45 Seq</i>	GCCACCTGGAGGTGATGAACAT
	Fw <i>lin-45 Seq</i>	GGCCCAGATAGATCAGTTGCCG
<i>csIs34</i> and <i>csIs42</i> genotyping	Fw <i>hsplin-45</i>	GAGGAAGCCAACACGCTTTG
	Rev <i>hsplin-45</i>	CAATTCGAAATGGCATCCCCG
14 words omitted		
<i>eri-1(mg366)</i> genotyping	Fw <i>eri-1(mg366)</i>	GATAAAACTTCGGAACATATGGGGC
	Rev <i>eri-1(mg366)</i>	ACTGATGGGTAAGGAATCGAAGACG
<i>lin-15(n744)</i> genotyping	Fw <i>lin-15b</i>	CTTCAGGACCATCACAGGATCG
	Rev <i>lin-15(n744)</i>	CGTCTGACGCATTCCCAAT
	Rev <i>lin-15(wt)</i>	CGTCTGACGCATTCCCAAC
<i>wpSi1</i> genotyping	Fw <i>Cbrunc119</i>	CCGAACAAGGAATCACAACGG
	Rev <i>Cbrunc119</i>	CCACAGTTGCTCCGACCG

<i>smn-1(cb131)</i> genotyping	Fw2 <i>smn-1(cb131)</i>	ACGGAGCTAATCAAAATGTATA
	Fw2 <i>smn-1(wt)</i>	ACGGAGCTAATCAAAATGTATG
	Rev <i>smnR4</i>	CCGCAATAGCTTCTTCATTC
17 words omitted		
18 words omitted		
<i>sid-1(qt9)</i> genotyping	Fw <i>sid-1(qt9) wt</i>	AAACCTGATCGTAACCGCGC
	Fw <i>sid-1(qt9) mut</i>	AAACCTGATCGTAACCGAGT
	Rev <i>sid-1(qt9) wt</i>	TTCGGGAAATGGCGCTTAAC

TABLE 4.3. **Primers used:** the table above shows the primers designed in *C. elegans* for the whole work. The application, the name of the primer and the sequences are reported.

4.8. Statistical Analysis

GraphPad Prism software was used for statistical analysis. The statistical significance was determined using a ONE-WAY ANOVA test with a non-parametric analysis (Kruskal-Wallis test) and Dunn's multiple comparison, or t-test non-parametric analysis and Mann-Whitney test compare rank. The experimental results are provided with standard error of the mean (SEM), and the total number of animals analyzed. The null hypothesis was rejected at the 0.05 level.

Backward locomotion assay analysis was performed as followed. I assigned 1 to each animal able to perform the backward movement, and 0 to each animal unable to fully move back. I performed the statistical analysis on this raw data on the total on the animal tested using a ONE-WAY ANOVA test with a non-parametric analysis (Kruskal-Wallis test) and Dunn's multiple comparison. Lifespan assays analysis was carried out by log-rank (Mantel-Cox) test with Gehan-Breslow-Wilcoxon test for multiple comparisons.

The spreading test statistical analysis were performed using iNumbers software (version 12.2.1). The statistical significance was determined using compare two proportion test. The null hypothesis was rejected at the 0.05 level.

CHAPTER 5

Mammals methods

5.1. Materials

Media and reagents for cell culture were obtained from Gibco® by Life Technologies, PAA Laboratories GmbH and VWR International. Chemicals for Biochemical and Molecular Biology methods were obtained from Sigma-Aldrich GmbH, Roche, Thermo Fisher Scientific, Bio Rad Laboratories Inc., NeoFroxx, Promega, Invitrogen.

5.2. Cell Cultivation

The murine hybrid cell line NSC34 cells (neuroblastoma × spinal cord neurons) (Cashman *et al.*, 1992) were cultured in proliferation medium consisting of DMEM 1× high glucose, supplemented with GlutaMAXTM-1, 10% FCS, 1% Penicillin/Streptomycin 5.000 U/L, at 37°C in a humidified atmosphere containing 5% CO₂.

For protein extraction and quantitation (see paragraph 5.3), 300,000 NSC34 wt and NSC34 20% cells were seeded in a 6-well plate. For protein extraction two wells were pooled for each condition tested. Twenty-four hours after seeding, the medium was replaced with “differentiation medium” consisting of starvation media made of DMEM 1× high glucose, supplemented with GlutaMAXTM-1, 1% FCS, 1% Penicillin/Streptomycin 5,000 U/mL. The cells were then differentiated for 72h in a humidified atmosphere with 5% CO₂ at 37°C. Cell lysis and protein extraction were performed when the cells reached ~80% confluence.

The human fibroblast cell lines, fibroblast Healthy ML35 and fibroblast Type 0, were maintained in proliferation medium made of (DMEM 1× high glucose, 20% FCS, 1% Penicillin/Streptomycin 5,000 U/mL) and incubated at 37°C in a humidified atmosphere containing 5% CO₂. For protein and RNA extraction and quantification (see paragraphs 5.3 and 5.4), 300,000 cells were seeded in a 10 cm Petri dish. To maximize the yield of both, protein and RNA extraction, two plates each condition were pooled. 96h after seeding, cells reached ~80% confluence and cell lysis, protein or RNA extraction were, then, performed.

Both cell lines were passed two times a week reaching a maximal confluency of 80 to 90 % before splitting.

5.2.1. Drug treatment

Fibroblasts Type 0 were treated with Evrysdi® (Risdiplam) for 24h, to evaluate 1 word omitted transcript and protein levels, and 1 word omitted transcript levels. Cells were seeded and maintained as previous described. 72h after seeding, cell reached ~60-70% confluence and they were treated with Evrysdi® (Risdiplam). To perform the 24-h treatment, the media was replaced

with fresh one supplemented with Evrysdi® (Risdiplam) diluted to a final concentration of 1µM. The next day, the plates are ~80% confluent and protein or RNA were extracted.

5.3. Protein extraction and Protein quantification

To analyze protein levels by Western Blot, proteins were extracted from cells or spinal cord of Taiwanese SMA and heterozygous mice and then quantified by Bicinchoninic acid (BCA) assay.

5.3.1. RIPA Cell Lysis

Cells from *in vitro* experiments were lysed to disrupt the cell membrane and then protein levels were determined in the samples. Protein degradation was prevented by the addition of proteinase and phosphatase inhibitors. Lysis was performed on ice. Cells were firstly washed with PBS at room temperature to remove residual dead cells and medium. The lysis mix consisting of RIPA buffer, PhosphoSTOP™ 20× (final concentration 1×) and cOmplete™ protease inhibitor cocktail 50× (final concentration 1×) was then added. For NSC34 lysis, 150 µL of Lysis Buffer was added to each sample and for fibroblasts, 300 µL of Lysis Buffer was added to each sample. Working on ice to enhance detaching, cells were scraped off with a cell scraper, collected in an Eppendorf tube and then incubated for 20' on ice (4°C). Samples were then further disrupted using a 1mL syringe and a G21 needle. The remaining cell fragments were pelleted by centrifugation at 21,000 g for 15' at 4°C. The supernatant was stored at -20°C and 6 µL was used to determine protein concentration in a BCA assay (see paragraph 5.3.3). 30 µg of NSC34 cell lysate and 10µg of Fibroblasts cell lysates were loaded on gel to evaluate protein and phospho-protein levels through Western Blot analysis.

5.3.2. Lysis of spinal cords from postnatal mice P3

Frozen spinal cord segments (spinal cord preparation L1-5 of postnatal mice P3) of postnatal mice were prepared as lysates to analyze protein levels by Western Blot analysis. The entire procedure was performed on ice. One spinal cord was lysed using a TissueLyser™ in a 2 mL reaction tube containing one tungsten carbide bead (3 mm) and 150 µL Lysis Mix (prepared as described in paragraph 5.3.1) at 50 Hz for 2.5'. Tissue debris was removed by centrifugation at 21,000 g for 30 min at 4°C. The supernatant was stored at -80°C and 6 µL of each sample was diluted in 54 µL PBS to measure the protein concentration using a BCA assay (see section 5.3.3). 20 µg of mice spinal cord lysates were loaded on gel to evaluate protein and phospho-protein levels through Western Blot analysis.

5.3.3. Bicinchoninic acid (BCA) assay

The amount of protein in a solution was determined using a BCA assay. The assay is based on the principle that copper ions (Cu^{2+}) can bind to peptide bonds in proteins. At an alkaline pH, copper ions are reduced to their ionic form (Cu^{+}), which forms a complex with bicinchoninic acid. This complex has a maximum absorption at 562 nm and the amount of absorbance is proportional to the protein concentration in the sample. The I prepared Standards, seven serial dilutions of 4 mg/mL BSA (bovine serum albumin) in PBS and samples (see paragraphs 5.3.1 and 5.3.2). A 96-well plate

was then prepared by adding 200 μ L of Pierce reagents (Reagent A and Reagent B) in a 50:1 ratio and 25 μ L of sample to each well. The plate was incubated at 37°C for 30' to accelerate the reaction and the excitation was measured using Gen5™ software in the BioTek™ Plate Reader at 562 nm. To determine protein concentration, a standard curve was constructed using the Standards, and the protein concentration in each sample was determined by comparing the absorbance to the standard curve. Samples were analyzed in duplicate and standards in triplicate. This assay was used to evaluate the protein concentration of both spinal cord lysates and cell culture lysates.

5.4. Western Blot analysis

To evaluate protein levels and phopho-protein, cells lysates or spinal cord lysates were applied on gel. First, samples were dissolved in 5× Laemmli buffer and heated at 95°C for 5' to denature proteins. Samples were, then, cooled down on ice immediately, and a second centrifugation step was performed to collect the condensed water. Then they were separated by SDS-PAGE. Proteins were blotted on a nitrocellulose membrane (0.45 μ m, GE Healthcare UK Ltd) for 1h at 120V. To evaluate the transfer efficacy and observe the proteins profile, after blotting the membrane was stained with Ponceau S. When the membrane was completely destained from Ponceau, it was blocked with blocking solution (5% BSA in TBS-Tween or 5% Milk in TBS-Tween), to prevent binding of unspecific antibodies. Then an overnight incubation at 4°C with the primary antibody was performed. Primary antibodies used were: 20 word2 omitted mouse α -SMN (1:2,000, monoclonal, BD Transduction Laboratories, #610647). Secondary antibodies ECL Anti-mouse IgG HRP linked F(ab)2 fragment (1:4,000, Cytiva NA931V) and ECL Anti-Rabbit IgG HRP linked whole antibody (1:5,000, Cytiva NA934V) were diluted in 5% milk powder in TBS-T, incubated for 1h at room temperature and used for detection of target proteins by chemiluminescence.

Stripping was performed to remove antibodies with western blot stripping buffer for 80' at 50°C.

Immunodetection for 1 word omitted was performed using Pierce™ ECL Western (Thermoscientific #32106); for 1 word omitted using SuperSignal™ West Femto Maximum Sensitivity Substrate (Thermoscientific #34096), and for SMN Immobilon Western Reagent Kit (Millipore #WBKLS0500). Densitometry was performed with the program LabImage 1D. The relative protein amount was calculated by the geometric mean over all samples for normalization and relativizing the proteins to the total amount of proteins obtained from the Ponceau Staining. Five biological replicates were tested.

5.5. RNA extraction and Real-Time PCR

Fibroblasts seeded on 10 cm Petri dishes were lysed to extract RNA using the NucleoSpin RNA Plus mini kit (Macherey-Nagel #740984.250). RNA quality check was assessed by running 1 μ L of each samples on an agarose gel to check for degradation. Reverse transcription was performed using M-MLV reverse transcriptase (Promega) and expression level of a desired gene was determined using SYBR Green PCR Master Mix + CXR (Promega) on the Step One Plus Thermocycler using the standard settings in Step One Software v2.3. Each sample was tested in technical duplicate and the housekeeping gene was tested in triplicate. Expression levels were

normalized to the average level of the housekeeping gene *hPPIA* by subtraction, then the relative change in transcript expression of each sample was determined by the geometric mean over all samples of each gene tested. Each experimental assay was performed in biological sextuplicate. The oligonucleotide sequences used for transcript analysis are listed in table 5.1.

Gene tested	Primer name	Sequence 5'⇒3'
<i>hSMN</i>	Forward 1082_hSMN_FL_3	CTGATGCTTGGGAAGTAT
	Reverse 1083_hSMN_FL_3	GCCAGCATTTCTCCTTAA
	Forward 1084_hSMN_D7_3	GTCCAGATTCTCTTGATGAT
	Reverse 1085_hSMN_D7_3	GCCAGCATTTCCATATAATAG
	Forward 1086_hSMN_ex6	GCTGATGCTTTGGGAAGTATGTTA
	Reverse 1087_hSMN_ex8	ATTCCAGATCTGTCTGATCG
9 words omitted		
9 words omitted		
9 words omitted		
9 words omitted		

TABLE 5.1. **Primers used for Real-Time PCR:** the table above shows the primers designed for the analysis of transcripts in human fibroblasts. The gene of interest, the name of the primers and their sequences are given.

5.6. Statistical Analysis

GraphPad Prism software was used for statistical analysis. The statistical significance was determined using a ONE-WAY ANOVA test with a non-parametric analysis (Kruskal-Wallis test) and Dunn's multiple comparison or using t test with non-parametric analysis (Mann-Whitney). The experimental results are provided with standard deviation (SD), and the total number of biological replicates analyzed. The null hypothesis was rejected at the 0.05 level.

Results

CHAPTER 6

Exploitation of a *C. elegans* SMA model to identify neuroprotective compounds

As mentioned above (see Chapter 1 paragraph 1.4.2), one of the modern strategies adopted to treat Spinal Muscular Atrophy is the development of SMN-independent treatments through the identification of small molecules and natural compounds. These substances, supplemented to diet as *functional foods* and added to current therapies, may prevent neurodegeneration or ameliorate neurodegenerative phenotypes, thanks to their low toxicity and their ability to act on multiple biochemical targets. In this context, the availability of model systems that allow rapid *in vivo* analysis, such as *Caenorhabditis elegans*, is crucial. Since *C. elegans* has proven to be a valid tool to perform drugs screenings and identify new neuroprotective molecules, such as 4-aminopyridine (4-AP) (Sleigh *et al.*, 2011) or natural compounds as green kiwi fruit extract (Mazzarella *et al.*, 2019), I adopted the only *C. elegans* SMA model useful to study neurodegeneration (Gallotta *et al.*, 2016). In this model I evaluated the effect of *Vigna unguiculata* (Cowpea Seed) natural extract on the neurodegenerative phenotype. In addition, I also validated some previous results on 4 compounds that have been identified as neuroprotective by a drug screening performed in the lab.

6.1. *Vigna unguiculata* protects from neurodegeneration caused by *smn-1* deficiency

Vigna unguiculata or cowpea, holds a prominent position among the *Vigna* species cultivated for human consumption. It thrives in tropical and subtropical regions worldwide, including Africa, Asia, Latin America, and selected Mediterranean countries (Tripodi *et al.*, 2020). Cowpea seeds are a valuable source of proteins, and of bioactive peptides with antioxidant activity. These features, coupled with the presence of minerals (calcium, iron and zinc) and phytochemicals, such as phenolic compounds, suggest potential health benefits of cowpea, including anti-diabetic, anti-cancer, anti-hyperlipidemic, anti-inflammatory and anti-hypertensive properties (Tripodi *et al.*, 2020).

It is well established that oxidative stress is a contributing factor to neuronal damage in various neurodegenerative diseases, including SMA. Moreover, a neuroprotective role of antioxidant natural extracts in SMA has been demonstrated. For example, green kiwi fruit extract has been demonstrated to rescue the neurodegenerative phenotypes shown in the *C. elegans* the *smn-1*(*MNs RNAi*) transgenic SMA model, (Mazzarella *et al.*, 2019). Thus, I wondered whether *Vigna unguiculata* aqueous extract could rescue neurodegeneration, in the same *C. elegans* SMA model (Gallotta *et al.*, 2016).

To demonstrate whether cowpea aqueous extract has a neuroprotective role on *smn-1(MNs RNAi)* SMA model, I treated animals *in solido* as previously described with two different concentrations of *Vigna unguiculata* aqueous extract, 0.2% w/v and 0.5% w/v (obtained as described in Materials and Methods). Then the neurodegeneration was analyzed when the animal reached the young adult stage (figure 6.1). Water was used as mock and Valproic acid as positive control (Gallotta *et al.*,2016).

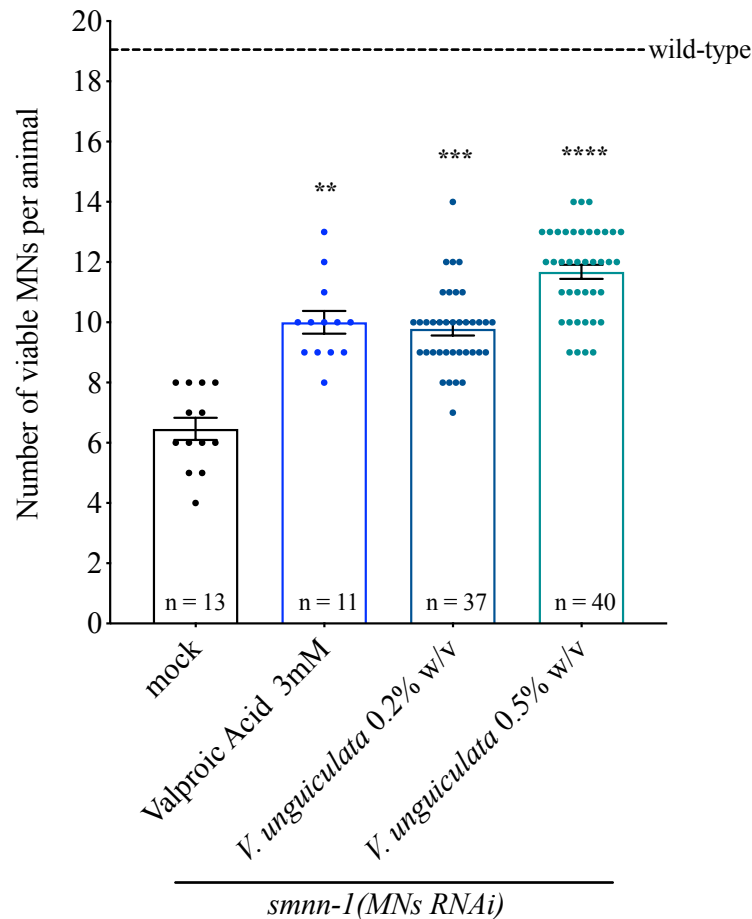


FIGURE 6.1. *Vigna unguiculata* aqueous extract rescues MNs degeneration caused by *smn-1* deficiency: the graph shows the mean of viable MNs per animal in *smn-1(RNAi)* untreated animals (mock: black bar), or treated with: Valproic acid 3mM (blue bar) as positive control (** $p = 0.0033$), *Vigna unguiculata* extract 0.2% w/v (dark blue bar) and 0.5% w/v (green blue bar). Cowpea seed extract partially rescues the neurodegeneration in a dose-dependent way compared to the mock: 0.2% w/v *** ($p=0.0007$), 0.5% w/v **** ($p<0.0001$) The motoneurons were identified as distinct GFP-expressing cells and quantified for each animal. The graph shows individual data points as scatterplots with bars and whiskers displaying mean and SEM. Each dot is the number of viable MNs in a single animal. The dashed line shows the number of viable MNs in wild-type animals (19). n is the total number of animals observed. One-way ANOVA with Kruskal-Wallis non-parametric analysis and Dunn's multiple comparison test was performed.

The analysis of the neurodegeneration showed that *Vigna unguiculata* aqueous extract is able to partially rescue the neurodegenerative defect caused by *smn-1* deficiency in a dose-dependent manner (figure 6.1). The mean of viable MNs after the treatment, indeed, increased from 6.46 of the untreated *smn-1(MNs RNAi)* animals to 9.78 of SMA animals treated with cowpea seed 0.2% w/v ($p = 0.0007$ versus mock) and to 11.67 of SMA animals treated with cowpea seed 0.5% w/v ($p<0.0001$ versus mock).

Although further studies are required to elucidate the molecular mechanism behind the neuroprotective effect and to identify the molecules of the extract involved in the neuroprotection,

this result further confirms the efficiency of *C. elegans* as a model organism for identifying neuroprotective compounds and supports the idea that incorporating antioxidant molecules into the diet could protect neurons from degeneration.

6.2. New compounds identified from a high-content drug screening protect from the neurodegeneration related to *smn-1* deficiency

The *smn-1* (*MNs RNAi*) *C. elegans* SMA model, valuable to study the mechanisms of MNs degeneration *in vivo*, has been in the last few years successfully used for an unbiased high-content drug screening, in collaboration with Prof. D. Medina, head of the High Content Screening Facility at TIGEM (Pozzuoli, Italy). The high content imager Operetta (Perkin Elmer) was used to acquire composite images of each single well from a 384-well microplate seeded with fixated *smn-1* (*MNs RNAi*) animals grown in liquid. Then, an appropriately developed script (Harmony software, Perkin Elmer) allowed to perform an automatic quantification of the mean of visible neurons/per animal/per well. *smn-1* (*MNs RNAi*) animals were treated with compounds from a Prestwick Library, composed of 1280 FDA-approved molecules, in order to identify and select neuroprotective compounds. Two rounds of automated screening have been carried out and 4 interesting neuroprotective compounds have been identified (Santonicola *et al.*, personal communication. In preparation).

To confirm the results obtained following the automated approach, and to possibly exclude false positives, I tested manually the four neuroprotective compounds *in liquido*. Animals were grown and treated *in liquido* as previously described. Each compound was tested at 100µM in DMSO 1% and DMSO 1% was used as mock, while PTZ (Pentylentetrazol) 100µM in DMSO 1% was the positive control. Neurodegeneration was scored when worms reached the young adult stage (figure 6.2).

The analysis of the neurodegenerative defect partially confirmed the screening result. Three out of the four identified compounds showed a neuroprotective activity. Compared to the mock with a mean of visible MNs of 5.17, Pimozide ($p<0.0001$), Compound 1 ($p<0.0001$) and Compound 2 ($p<0.0001$) increased this value, showing a mean of visible MNs equal to 8.07, 7.35 and 6.74, respectively. In contrast, animals treated with Compound 3 showed a mean of visible MNs of 5.80, a value not statistically different from the mock.

These results demonstrate the efficacy of *C. elegans* as a tool to perform automated drug screening to identify new neuroprotective compounds. This validation experiment allowed to confirm 3 compounds identified, Pimozide, Compound 1 (patent application to be compiled) and Compound 2 (patent application to be compiled) and to exclude Compound 3 being a false positive. To further support the validity of our approach, Pimozide has been recently published to be effective in another SMA *C.elegans* model (Doyle *et al.*, 2020), hence disclosed. Further investigations are required to elucidate the mechanism of action of these compounds in neuroprotection, define if their mechanism of action is SMN-independent, and determine whether their neuroprotective effect is conserved in mammals to confirm a putative role of these candidates as future treatments for SMA.

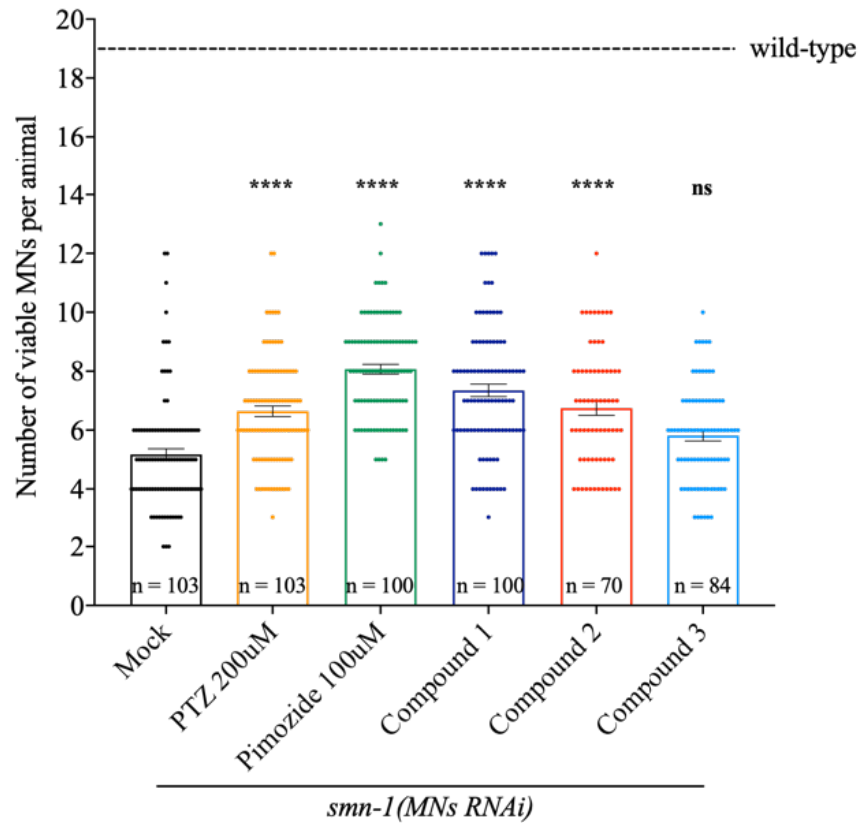


FIGURE 6.2. New neuroprotective compounds able to rescue the neurodegenerative defect caused by *smn-1* deficiency: the graph shows the mean of viable MNs per animal in *smn-1(RNAi)* untreated animals (mock: black bar), or treated with PTZ (orange bar) as positive control (**** $p < 0.0001$ vs mock), or treated with the four compounds identified following the semi-automated screening. The motoneurons were identified as distinct GFP-expressing cells and quantified for each animal. Pimozide (green bar), Compound 1 (blue bar) and Compound 2 (red bar) partially rescue the neurodegeneration caused by *smn-1* deficiency compared to the mock (**** $p < 0.0001$). Compound 3 (light blue bar) doesn't affect the neurodegenerative defect ($p = 0.2439$). The graph shows individual data points as scatterplots with bars and whiskers displaying mean and SEM. Each dot is the number of viable MNs in a single animal. The dashed line shows the number of viable MNs in wild-type animals (19). n is the total number of animals observed. One-way ANOVA with Kruskal-Wallis non-parametric analysis and Dunn's multiple comparison test was performed.

CHAPTER 7

***B-Raf/lin-45* contributes to motoneuron degeneration in SMA**

The development of new therapeutic strategies for SMA which may complement the existing ones, might require the identification of new systems and pathways affected in the disease. This approach, through the elucidation of the molecular basis of the disorder, would enable the development of therapeutic approaches that may lead to a definitive cure for this devastating disease. In this regard, as mentioned in the previous chapters, network biology offers an efficient alternative approach to understand the interactions between proteins involved in dysregulated signaling pathways, in order to select new attractive drug targets.

The phospho-array and network biology approach developed by Prof. Claus' group proved effective and resulted in the identification of B-RAF as a new SMA modifier (Hensel *et al.*, 2021). The phospho-array results were confirmed by western blot analysis in Taiwanese SMA mice Spinal Cord (see Chapter 1 paragraph 1.5) and B-RAF ortholog role in SMA pathway and neurodegeneration was studied *in vivo* in *C. elegans* (see Chapter 3 paragraph 3.4). In the following paragraphs are reported further results that I obtained regarding *B-RAF/lin-45* characterization *in vivo*. These results confirm that the neurodegenerative events associated with SMA, in which *B-RAF/lin-45* is involved, are conserved across species and provide important information on the mechanism of action of its neuroprotective effect (Hensel *et al.*, 2021). Building upon the findings presented in Chapter 1 (paragraph 1.5) and Chapter 3 (paragraph 3.4), the results here described have also been published in Hensel *et al.*, 2021.

7.1. *B-RAF/lin-45* is pre-symptomatically down-regulated in the *C. elegans* severe SMA model

The degeneration of motoneurons as a result of SMN loss is conserved across a wide range of organisms, including *C. elegans*. As mentioned above (see Chapter 3 paragraph 3.4), the MAPK/ERK signalling pathway is perfectly conserved, with the *B-RAF* ortholog *lin-45* being expressed in both neurons and motoneurons. The phospho-array experiment performed on the spinal cord of SMA Taiwanese mice showed a down-regulation of B-RAF protein levels in SMA compared to control, and this result was confirmed in spinal cord and patients' fibroblasts by Western blot (see Chapter 1 paragraph 1.5). Thus, to further investigate the role of *B-RAF/lin-45* in neurodegeneration *in vivo*, I first hypothesized that the reduction of *B-RAF/lin-45* in response to *SMN/smn-1* deficiency is conserved also in *C. elegans*. To demonstrate that the dysregulation of *B-RAF/lin-45* related to *SMN/smn-1* deficiency is conserved in nematodes, and to confirm that *C. elegans* is an useful model to elucidate the *B-RAF/lin-45* role in neurodegeneration *in vivo*, the *lin-45* transcript levels were quantified. Particularly, to mimic the conditions used in phospho-array experiment (see Chapter 1),

I evaluated *lin-45* transcript levels in the most severe *C. elegans* SMA model, the *smn-1(ok355)* knockout strain (see Chapter 3 paragraph 3.3.2). The transcript levels of this *lin-45* gene were quantified at L2 larval stage, when animals do not show any deleterious SMA related phenotype, to simulate the pre-symptomatic condition (figure 7.1) (Hensel *et al.*, 2021).

In these pre-symptomatic animals, it was observed a 78% reduction in *lin-45* transcript levels in *smn-1* knockout compared to wild type (figure 7.1), thus recapitulating the observations in SMA mice spinal cords and patient fibroblasts, and confirming that *lin-45* expression is modulated by *smn-1*. Since is very difficult to work with those animals due to their pleiotropic phenotypes and early lethality (Briese *et al.*, 2009), to further characterize *lin-45* effect on *smn-1* associated neurodegeneration I moved to the SMA model where *smn-1* is silenced only in motoneurons (Gallotta *et al.*, 2016).

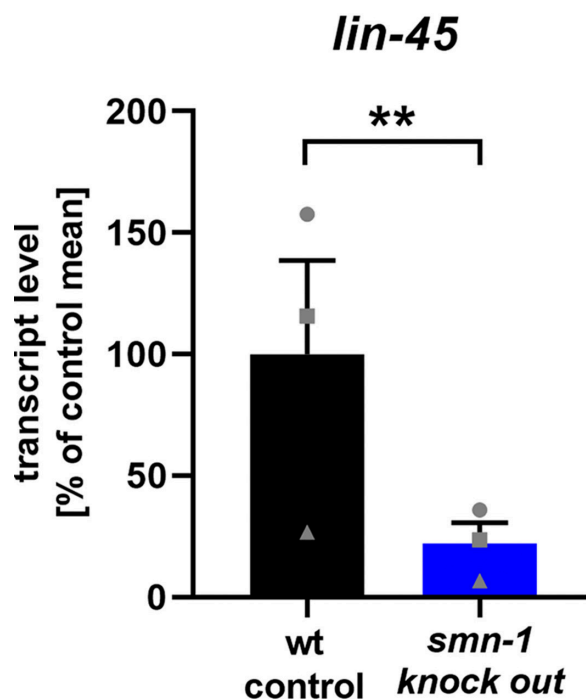


FIGURE 7.1. *lin-45* transcript levels are reduced in *smn-1* knock-out pre-symptomatic animals: the graph shows the quantitative analysis of *lin-45* expression by qPCR in *C. elegans* *smn-1* knock-out mutant (blue bar), *smn-1(ok355)*, compared to wild type (black bar). Three biological independent replicates were performed in a paired experimental setup. Graph shows individual data points as scatterplots with bars and whiskers displaying mean and SEM. Paired-ratio Student's t test was performed: ** $p \leq 0.01$ (Hensel *et al.*, 2021).

7.2. *B-RAF/lin-45* partially rescues motoneuron degeneration in a *C. elegans* SMA model

B-RAF and its ortholog *lin-45* are expressed in neurons as well as in motoneurons. Furthermore, the phospho-array and western blot results demonstrated that B-Raf protein levels and phospho-B-Raf protein levels are strongly reduced in SMA mice spinal cord, supporting the idea that in SMA B-Raf protein is both less expressed and less active. Thus, a cell autonomous mechanism of *B-Raf/lin-45*-related motoneuron degeneration was hypothesized. To demonstrate the cell-autonomous neuroprotective role of *B-Raf/lin-45*, I performed a cell-specific rescue approach in *smn-1(MNs)*

RNAi) model of SMA (Gallotta *et al.*, 2016). A subset of motoneurons in those animals degenerate and can be monitored by the expression of green fluorescent protein (GFP) (Gallotta *et al.*, 2016). Specifically, four transgenic lines expressing *lin-45* in MNs were created in *smn-1(MNs RNAi)* genetic background and then I quantified the number of visible MNs in these animals (figure 7.2). Two transgenic lines over-express the wild-type *lin-45* under the motoneuron specific *punc-47* promoter (*MN-lin-45^{wt}*) at two different concentrations: a lower one (*MN-lin-45^{wt}LC*) and an higher one (*MN-lin-45^{wt}HC*). The same promoter was, then, also used to obtain the other two transgenic lines over-expressing a hyperactive form of *lin-45* (*MN-lin-45^{hyp}*) at a lower (*MN-lin-45^{hyp}LC*) and a higher concentration (*MN-lin-45^{hyp}HC*) (Hensel *et al.*, 2021). The *lin-45* hyperactive form carries the same mutation S312A previously described (see Chapter 3 paragraph 3.4), encoding for a protein lacking an inhibitory phosphorylation site, which causes its hyperactivation (Kao *et al.*, 2004).

The analysis of the neurodegeneration confirmed previous results. The *smn-1* silencing by motoneuron-specific RNAi induced motoneuron loss, with a mean of viable MNs in *smn-1(MNs RNAi)* animals of 7.36, while the mean of viable MNs in wild-type animals expressing GFP in MNs is 18.61 (figure 7.2 A and B). Co-expression of wild-type (*MN-lin-45^{wt}*) or hyperactive *lin-45* (*MN-lin-45^{hyp}*) in motoneurons using a higher concentration (HC) partially rescued motoneurons loss to similar levels, with a mean of viable MNs of 11.03 and 11.09, respectively. On the other hand, only *lin-45^{hyp}* at lower concentration (LC) slightly, but significantly, rescued the neurodegeneration (figure 7.2 A and B), and the mean of visible MNs in this strain is 9.54 (statistically different from the *smn-1(MNs RNAi)* strain, $p < 0.0001$) (Hensel *et al.*, 2021).

These results indicate a cell autonomous *lin-45* neuroprotective role strongly related both to *lin-45* expression levels and protein activity.

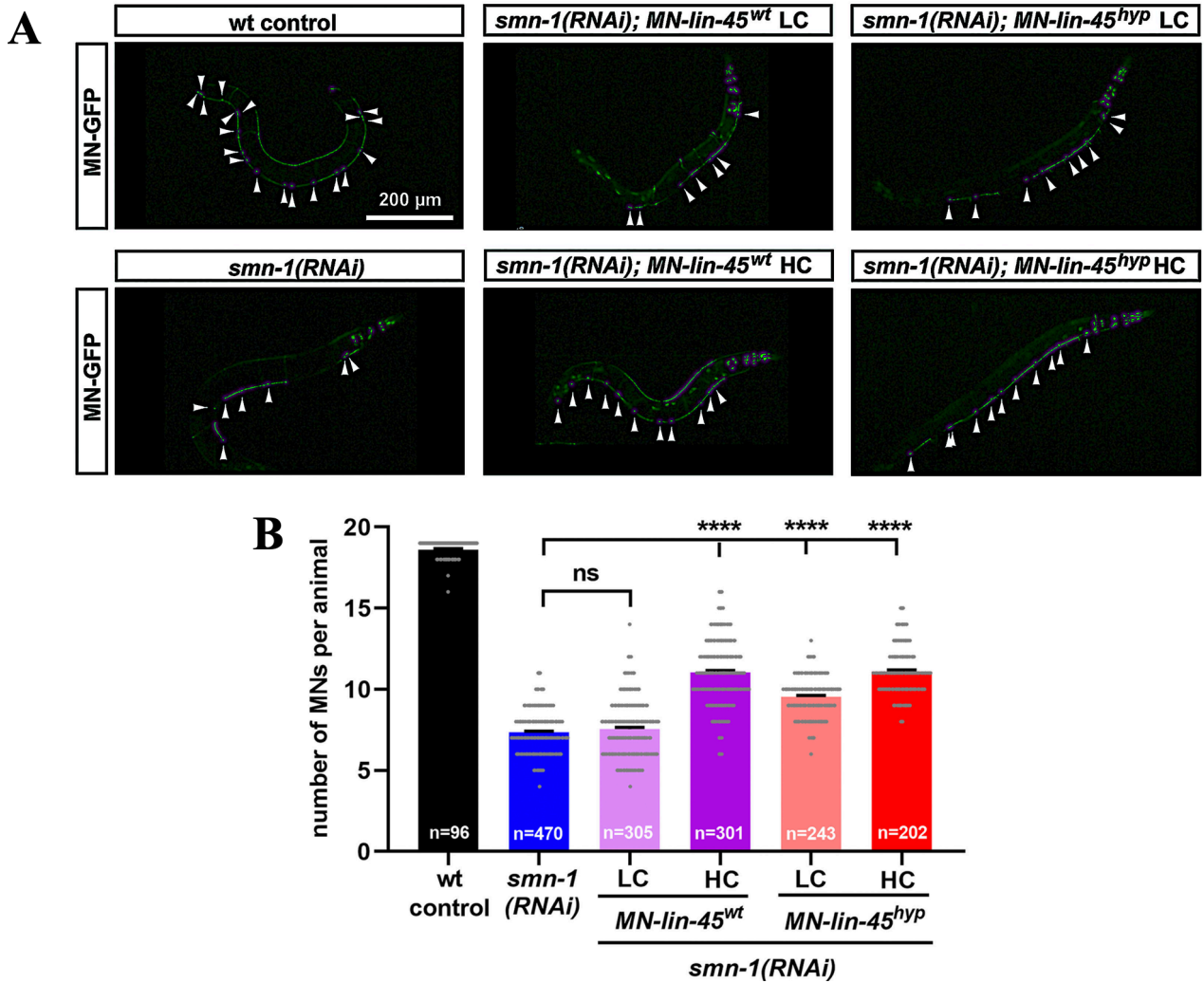


FIGURE 7.2. *lin-45* cell-autonomous neuroprotective role in MNs: **A.** Representative images of *C. elegans* animals expressing GFP under the *punc-25* promoter, which exclusively drives expression in D-type motoneurons (MN-GFP). Arrowheads show individual D-type motoneurons. *smn-1* was silenced in D-type motoneurons: *smn-1(RNAi)*. Wild-type animals served as controls (wt control). The motoneuron promoter *punc-47* drives the expression of *lin-45* either as a wild type (*MN-lin-45^{wt}*) or as a hyperactive mutant (*MN-lin-45^{hyp}*) at low and high concentrations (LC or HC). **B.** The motoneurons were identified as distinct GFP-expressing cells and quantified for each animal. The graph shows the mean of viable MNs per animal in wt control animals, expressing GFP in MNs (black bar); in *smn-1(RNAi)* animals (blue bar); in *smn-1(RNAi)* animals overexpressing *lin-45^{wt}* in MNs at lower (light purple bar) and higher concentrations (purple bar); in *smn-1(RNAi)* animals overexpressing *lin-45^{hyp}* in MNs at lower (pink bar) and higher concentrations (red bar). The neurodegenerative defect was partially rescued when *lin-45^{wt}* was expressed at higher concentration (**** $p < 0.0001$) and when *lin-45^{hyp}* was expressed at both concentrations tested (**** $p < 0.0001$). The graph shows individual data points as scatterplots with bars and whiskers displaying mean and SEM. Each dot is the number of viable MNs in a single animal. n is the total number of animals observed. One-way ANOVA with Kruskal-Wallis non-parametric analysis and Dunn's multiple comparison test was performed (Hensel *et al.*, 2021).

7.3. *B-RAF/lin-45* partially rescues also motor function in a *C. elegans* SMA model

lin-45 plays a role in locomotion (Coleman *et al.*, 2018) and the *smn-1(MNs RNAi)* SMA model exhibits a defect in backward locomotion, because D-type MNs are involved in the regulation of this behavior. Thus, I investigated whether the cell-autonomous neuroprotective effect mediated by *lin-45* was also conserved at functional level, rescuing the impairment in backward locomotion as well. To this end, I performed a backward locomotion assay in transgenic strains over-expressing in *smn-1(MNs RNAi)* genetic background: *MN-lin-45^{wt} LC*, *MN-lin-45^{wt} HC*, *MN-lin-45^{hyp} LC* and *MN-lin-45^{hyp} HC* (figure 7.3) (Hensel *et al.*, 2021).

The analysis of backward locomotion confirmed that the neuroprotective effect of *lin-45* is also evident at the functional level. The locomotion defect observed in *smn-1(MNs RNAi)* strain, in which only 39% of animals showed a normal locomotion, were partially rescued by the hyperactive *lin-45* form at both concentrations tested, and slightly, but not reaching statistical significance, rescued by *lin-45^{wt}* expression at high concentration (figure 7.3).

Together these results suggest a cell-autonomous conserved role of *lin-45* in motoneurons and that *lin-45* reduction is a one of the major causes for motoneuron degeneration and dysfunction observed after *smn-1* silencing.

7.4. *lin-45* partially rescues motoneurons degeneration in symptomatic animals through the MAPK Pathway

B-Raf/lin-45 shows a cell-autonomous neuroprotective effect when *SMN/smn-1* is lacking. To investigate the downstream molecular mechanism of this neuroprotective effect, some of the *B-RAF/lin-45* downstream targets were tested. To this end I employed a heat-inducible S312A mutated strain (*i-lin-45^{hyp}*) that expresses an inducible form of *lin-45* (Hensel *et al.*, 2021) and then I performed a pharmacological and a genetic approach.

7.4.1. Heat-induction allows *i-lin-45^{hyp}* expression

As previously described (see Chapter 3 paragraph 3.4), similar to the cell autonomous *lin-45* restoration, heat-induced *i-lin-45^{hyp}* partially rescued motoneurons loss and motor functions in animals with MNs-specific *smn-1* knockdown (figure 3.9 A and B), whereas the same result wasn't observed following *i-lin-45^{wt}* heat-induction. To find a possible explanation to this finding and select the most efficient model to further investigate the molecular basis of the neuroprotection *lin-45* mediated, at first I assessed the *lin-45* transcript levels in heat-inducible strains (figure 7.4).

The transcript level analysis showed that the only strain able to express *lin-45* when maintained at 25°C is the *i-lin-45^{hyp}* one, suggesting a successful triggering of the heat-inducible promoter. Unexpectedly, there was no substantial expression of *lin-45* from the wild-type construct (*i-lin-45^{wt}*) compared to the hyperactive variant and the wild-type (figure 7.4). This explains why the constitutive expression of *lin-45^{wt}* didn't rescue the neurodegenerative defect caused by *smn-1* silencing and supporting the dose-dependent neuroprotective effect of this new *smn-1* modifier.

7.4.2. *MEK/mek-2* and *ERK/mpk-1* inhibition blocks the *B-RAF/lin-45* neuroprotective effect in symptomatic animals

To investigate the downstream mechanisms of the *lin-45* rescue effect on neurodegenerative phenotypes caused by *smn-1* deficiency, the *i-lin-45^{hyp}* strain was selected to maximize *lin-45* activity and establish a robust and inducible rescuing system. I maintained this strain from the previous generation at induction temperature and treated it with the MAPK pathway inhibitors U0126 (*MEK/mek-2* inhibitor) and FR180204 (*ERK/mpk-1* inhibitor). Then the number of visible MNs at the young adult stage was evaluated. Inhibitors were applied at the L2 stage to reduce general toxic effects and to prevent any interference with neurogenesis (Hensel *et al.*, 2021).

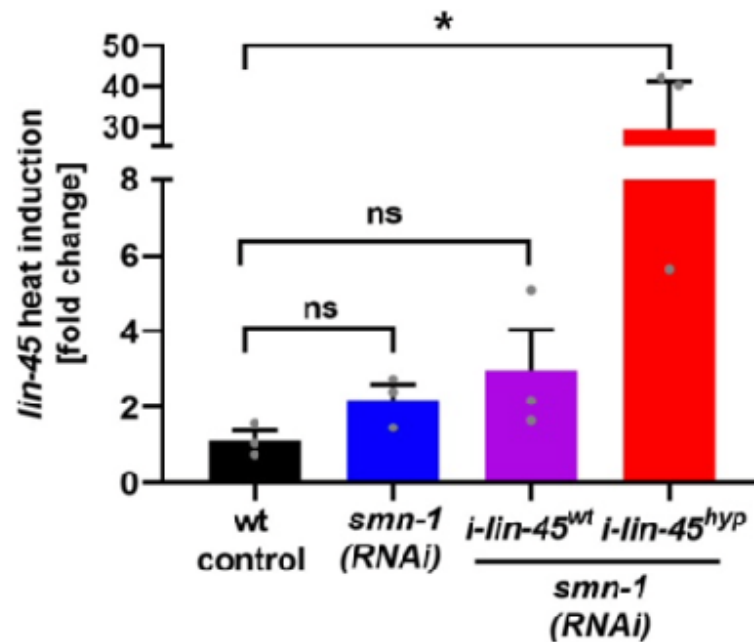


FIGURE 7.4. *i-lin-45* transcript levels following heat-inducible expression: the graph shows the *lin-45* transcript levels in wt control animals (black bar), *smn-1*(MNs RNAi) animals (blue bar), and animals expressing in *smn-1*(MNs RNAi) genetic background: *i-lin-45*^{wt} (purple bar) and *i-lin-45*^{hyp} (red bar). The fold change of the *lin-45* expression after temperature-induction is shown (25°C expression divided by 15°C expression). Only *i-lin-45*^{hyp} was highly expressed after the heat-induction (* p = 0.01). The graph shows individual data points as scatterplots with bars and whiskers displaying mean and SEM. Each dot represents the *lin-45* expression level in a single biological replicate. Three biological replicates were tested. One-way ANOVA with Kruskal-Wallis non-parametric analysis and Dunn's multiple comparison test was performed (Hensel *et al.*, 2021).

First, I assessed whether treatment in wild-type or *smn-1*(MNs RNAi) animals did lead to any reduction in the number of visible MNs (figure 7.5). This showed that *MEK/mek-2* or *ERK/mpk-1* inhibition does not affect cell survival or neurodegeneration, since the mean number of visible MNs in treated animals was not statistically different from untreated animals (figure 7.5) (Hensel *et al.*, 2021).

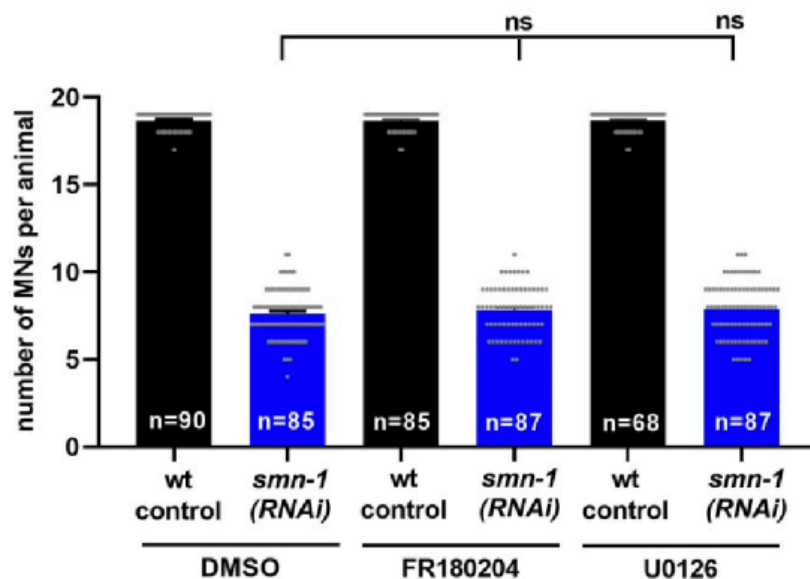


FIGURE 7.5. *MEK/mek-2* or *ERK/mpk-1* inhibition does not affect the number of viable MNs in wild-type and *smn-1*(MNs RNAi) animals: the graph shows the number of visible MNs of wild-type controls animal in which MNs are tagged with GFP (black bars) and of *smn-1* (MNs RNAi) animals (blue bars), after the treatment with FR180204 (*ERK/mpk-1* inhibitor), U0126 (*MEK/mek-2* inhibitor) or in untreated conditions (DMSO). The motoneurons were identified as distinct GFP-expressing cells and quantified for each animal. The graph shows scatterplots with bars and whiskers displaying mean and SEM. Each dot represents the number of visible MNs counted per each animal tested. n is the number of analyzed animals. One-way ANOVA with Kruskal-Wallis non-parametric analysis and Dunn's multiple comparison test was performed (Hensel *et al.*, 2021).

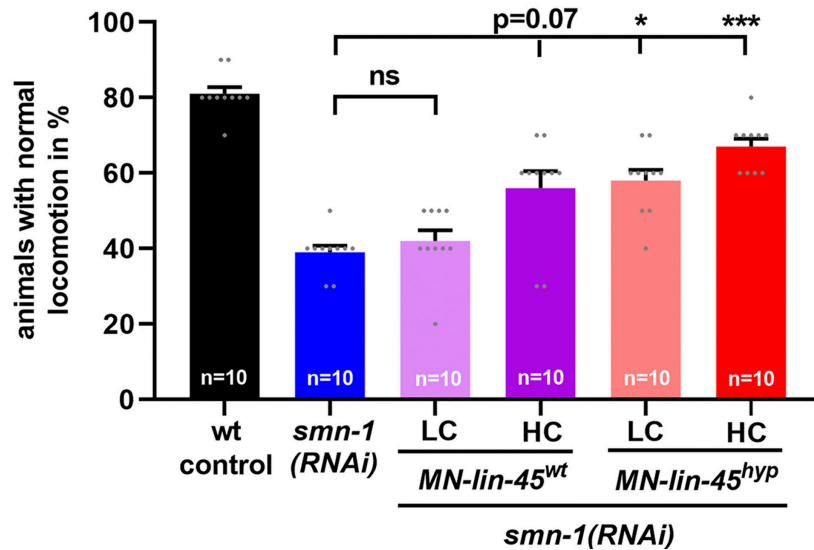


FIGURE 7.3. *lin-45* cell-autonomous neuroprotective role is also visible at functional level: the graph shows the percentage of animals tested for backward locomotion that are able to correctly perform this movement. The animal tested were wt control animals, (black bar); *smn-1(RNAi)* animals (blue bar); *smn-1(RNAi)* animals overexpressing *lin-45^{wt}* in MNs at lower (light purple bar) and higher concentrations (purple bar); *smn-1(RNAi)* animals overexpressing *lin-45^{hyp}* in MNs at lower (pink bar) and higher concentrations (red bar). The locomotion defect was partially rescued only when *lin-45^{hyp}* was expressed both at lower (* p = 0.01) and higher concentrations (*** p = 0.0005). The graph shows individual data points as scatterplots with bars and whiskers displaying mean and SEM. Each dot represents the percentage of animals among 10 tested with normal locomotion. n is the amount of dot represented and 100 animals for each genotype have been tested. One-way ANOVA with Kruskal-Wallis non-parametric analysis and Dunn's multiple comparison test was performed (Hensel *et al.*, 2021).

Then, I evaluated the drug inhibition effect on animals expressing *lin-45^{hyp}* in *smn-1(MNs RNAi)* genetic background (figure 7.6).

Inhibition of the *lin-45* downstream targets *mek-2* or *mpk-1*, completely abrogated the *i-lin-45^{hyp}* rescuing effect on *smn-1(RNAi)* (figure 7.6). Indeed, compared to the mean of visible MNs of untreated animals expressing *i-lin-45^{hyp}* in *smn-1(MNs RNAi)* genetic background (13.67), the mean of visible MNs of treated animals with the same genotype decreased to 9.31 when treated with *ERK/mpk-1* inhibitor (FR180204), and to 9.2 when treated with *MEK/mek-2* inhibitor (U0126). These values are more similar to the mean of visible MNs of the *smn-1(MNs RNAi)* animals (9.01), strongly support the conclusion that *lin-45* exerts its neuroprotective effect through the MAPK/ERK pathway.

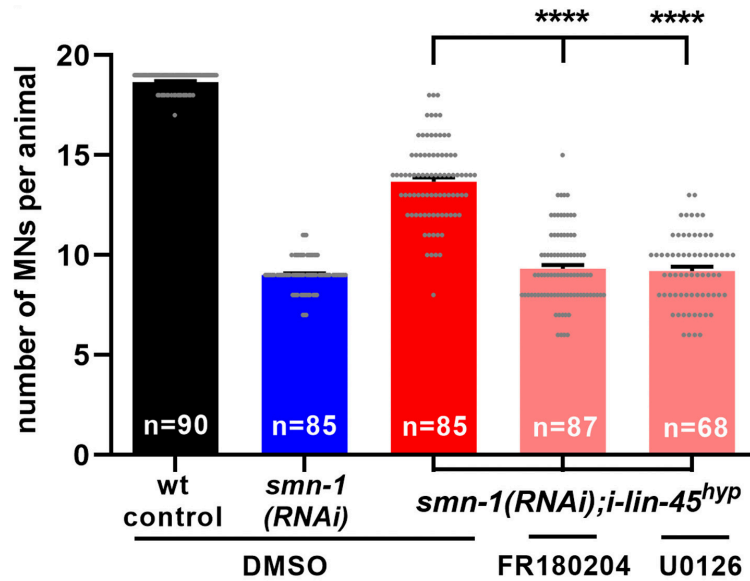


FIGURE 7.6. **MEK/*mek-2* and ERK/*mpk-1* inhibition abrogated the *i-lin-45^{hyp}* rescuing effect:** the graph shows the number of visible MNs of wild-type control animal in which MNs are tagged with GFP (black bars), of *smn-1 (MNs RNAi)* animals (blue bars), and of *smn-1(MNs RNAi)* animals expressing *i-lin-45^{hyp}* after the treatment with FR180204 (*ERK/mpk-1* inhibitor), U0126 (*MEK/mek-2* inhibitor) (pink bars) or untreated (DMSO, red bar). The motoneurons were identified as distinct GFP-expressing cells and quantified for each animal. Both FR180204 (**** $p < 0.0001$) and U0126 (**** $p < 0.0001$) treatments abolish the rescuing effect obtained with *lin-45*. Scatterplots with bars and whiskers display mean and SEM. Each dot represents the number of visible MNs counted per each animal tested. n is the number of analyzed animals. One-way ANOVA with Kruskal-Wallis non-parametric analysis and Dunn's multiple comparison test was performed (Hensel *et al.*, 2021).

7.4.3. *MEK/mek-2* loss of function mutant abrogates the *B-RAF/lin-45* neuroprotective effect

A pharmacological treatment could have some limitations, mainly related to the specificity of its effect. Thus, to further confirm the results obtained with *MEK/mek-2* and *ERK/mpk-1* inhibitors, I also employed a genetic approach. Specifically, I analyzed the neurodegeneration of a triple mutant expressing a *mek-2* loss of function mutation together with *smn-1(MNs RNAi)* and *i-lin-45^{hyp}* (figure 7.7). Since *mek-2* knockout is lethal, the *n1989* hypomorph mutant was used instead. *n1989* is a viable weak allele consisting in a S217F substitution (Kornfeld *et al.*, 1995).

The analysis of the neurodegeneration confirmed previous results with pharmacological inhibition (figure 7.6), and the *lin-45* rescue was abrogated by the *n1989* mutation in *mek-2* (figure 7.7). This demonstrates that the rescue of motoneuron degeneration in *smn-1(RNAi)* animals is mediated by the *mek-2/mpk-1* or MEK/ERK signaling cascade.

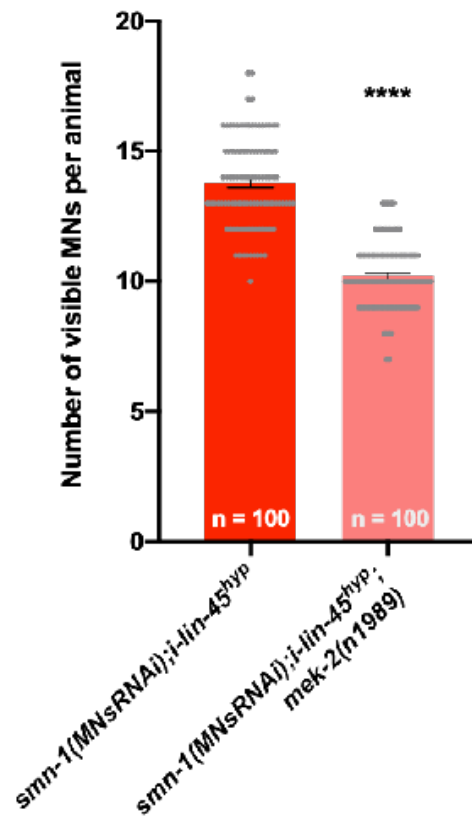


FIGURE 7.7. ***mek-2(n1989)* abrogated the *i-lin-45^{hyp}* rescuing effect:** the graph shows the number of visible MNs of *smn-1(MNs RNAi)* animals expressing *i-lin-45^{hyp}* (red bar) and *smn-1(MNs RNAi)* animals expressing *i-lin-45^{hyp}* and mutated in *mek-2 (n1989)* (pink bar). The motoneurons were identified as distinct GFP-expressing cells and quantified for each animal. *mek-2(n1989)* abolishes the rescue effect performed by *i-lin-45^{hyp}*. Scatterplots with bars and whiskers display mean and SEM. Each dot represents the number of visible MNs counted per each animal tested. n is the number of analyzed animals. *t-test* non-parametric analysis and Mann-Whitney test compare rank were performed (**** $p < 0.0001$) (Hensel *et al.*, 2021).

All these results, recently published (Hensel *et al.*, 2021), demonstrated that phospho-array approach and network biology are powerful tools to identify new target genes involved in SMA and encouraged us to explore the other candidates of the network. To this end, an RNAi screening of the *C. elegans* homologs of the 37 remaining proteins of the network has been performed in the last years.

CHAPTER 8

RNAi *by feeding* screening set up

The phospho-array approach developed by Hensel *et al.*, allowed the identification of 38 dysregulated proteins in Spinal Muscular Atrophy arranged in two clusters connected by a main hub represented by B-RAF/*lin-45*, whose role in the disease has been confirmed in mammals (see Chapter 1 paragraph 1.5) and characterized in *C. elegans* (see Chapter 3 paragraph 3.4 and chapter 7).

Since *C. elegans* has proven to be a perfect complementary model organism for the identification of new SMA modifiers, it was chosen to study the entire network in order to identify and select the most interesting dysregulated proteins for further studies. The quickest way to analyze a large number of candidates is by forward genetic screening, so I designed an RNAi *by feeding* screening to silence the *C. elegans* genes homologs to the dysregulated candidates of the network. To this purpose the *smn-1(MNs RNAi)* SMA model was adopted and the neurodegeneration was evaluated as phenotype.

The design of the screening system took into account the need to:

- obtain a strain that allows the silencing of candidate genes ubiquitously, including also neurons that are normally refractory to RNAi (Conte *et al.*, 2017), in order to select candidates that play their role also in nervous system;
- identify control genes for the screening from known suppressor and enhancer genes;
- identify modifiers capable of: i) rescuing the neurodegeneration caused by *smn-1* silencing, thus suppressing the neurodegeneration (*suppressor genes*); or ii) worsening it, thus enhancing the neurodegeneration (*enhancer genes*);
- obtain other strains that will allow to collect additional information on the candidates genes by performing tissue-specific silencing to identify in which tissue the candidates play their role;
- speed up the analysis.

8.1. Optimizing our SMA *C. elegans* model

Since the RNAi machinery is less active in nervous system and neurons are refractory to silencing (Conte *et al.*, 2017), to efficiently test the 37 proteins belonging to the network obtained through the phospho-array, and identify genes that play their role also in neurons, I optimized the *smn-1(MNs RNAi)* SMA model in order to hyper-sensitize the whole worm body, including the nervous system, to RNAi mediated silencing.

Thus, I obtained a triple mutant by genetic crosses. This new strain expresses in *smn-1(MNs RNAi)* genetic background, the mCherry protein in MNs to label these cells and, thus, quantify the neurodegeneration; and *eri-1(mg366)*, *lin-15b(n744)* loss of function mutations. Strains carrying these two mutations are synergistically sensitive to RNAi, increasing this sensitivity mainly in the nervous system (Wang *et al.*, 2005). ERI-1 is an exonuclease that antagonizes in neurons the RNAi

by degrading siRNAs (Grishok, 2005). The *mg366* allele consists in a 23 bp (base pairs) insertion encoding for seven amino acids followed by a premature stop codon that impairs protein activity promoting, as consequence, silencing events (Kennedy *et al.*, 2004). In addition, *lin-15b(n744)* mutant increases the sensitivity of all tissues, including neurons, to dsRNA that triggers the RNAi machinery (Calixto *et al.*, 2010). From the same genetic cross were also obtained the following sibling strains: animals hypersensitive to RNAi in all cells including neurons [*eri-1(mg366); lin-15b(n744)*] expressing the mCherry protein in GABA MNs to label these cells; animals silencing *smn-1*(MNs RNAi) and expressing the mCherry protein in GABA MNs; and animal only expressing the mCherry protein in GABA MNs.

First, I compared the neurodegeneration of this new optimized SMA models to the previous one (figure 8.1).

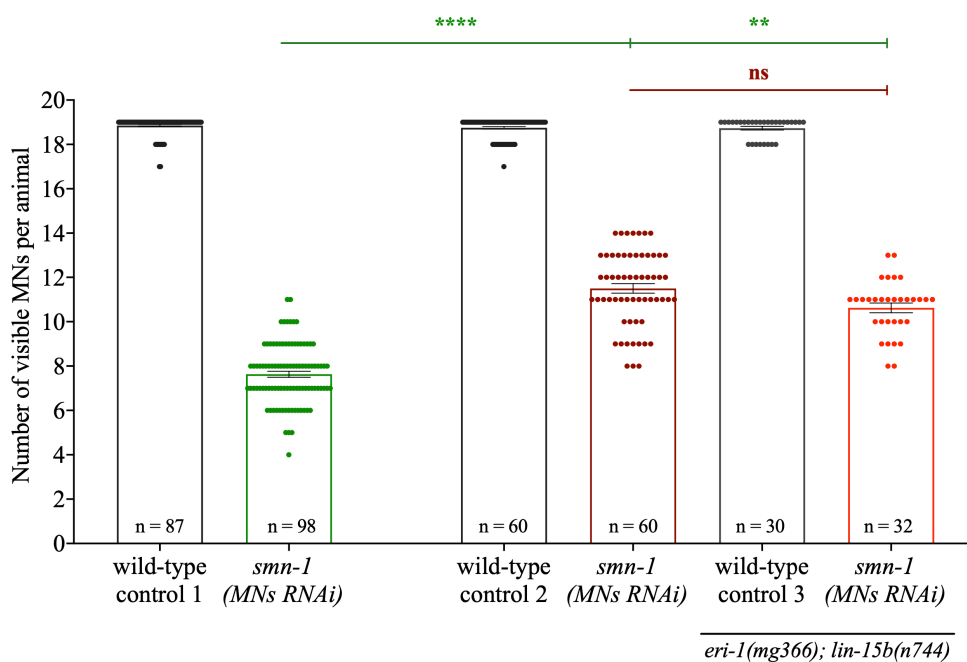


FIGURE 8.1. Score of the new SMA models optimized for RNAi screening: the graph shows on the left the number of visible MNs in RNAi non-hypersensitive strains with MNs tagged with GFP: wild-type control 1 animals (black bar), *smn-1* (MNs RNAi) animals (green bar). Then in the middle animals with MNs tagged with mCherry and RNAi non-hypersensitive strains are shown: wild-type control 2 animals (dark grey bar), and *smn-1*(MNs RNAi) animals (dark burgundy bar). Finally, on the right RNAi hypersensitive strains mutated in *eri-1(mg366); lin-15b(n744)* with MNs tagged with mCherry are shown: wild-type control 3 animals (grey bar) and *smn-1*(MNs RNAi) animals (red bar). The motoneurons were identified as distinct GFP/mCherry-expressing cells and quantified for each animal. All new *smn-1*(MNs RNAi) strains show a less severe neurodegenerative phenotype than the original one (green bar). Graphs are scatterplots with bars and whiskers displaying mean and SEM. Each dot represents the number of visible MNs counted per each animal tested. n is the number of analyzed animals. One-way ANOVA with Kruskal-Wallis non-parametric analysis and Dunn's multiple comparison test was performed. **** (p<0.0001), ** (p=0.0022), ns (p>0.9999).

The analysis of neurodegeneration shows that the number of visible MNs in the new control strains is not affected in either the RNAi non-hypersensitive (mean visible MNs 18.75) (figure 8.1 dark grey bar) or RNAi hypersensitive (mean visible MNs 18.73) (figure 8.1 grey bar) genetic background. On the other hand, both the new SMA strains not hypersensitive to RNAi in all cells (figure 8.1 bordeaux bar) and the new SMA strain hypersensitive to RNAi in all cells including neurons (figure 8.1 red bar) show the expected reduction in the number of visible MNs compared to their controls. The mean of visible MNs is indeed 11.5 and 10.62 respectively. Interestingly,

possibly due to the new genetic background, both new SMA strains (Figure 8.1 reddish bars) show a less severe neurodegenerative phenotype compared to the previously SMA model I used (Figure 8.1 green bar, $p < 0.0001$ vs RNAi non-hypersensitive SMA strain and $p = 0.0022$ RNAi hypersensitive SMA strain). This new trait can be easily genetically modulated in both directions thus, for the first time, we obtained a new model that allows us to identify modifiers that suppress or enhance the neurodegenerative phenotype.

8.1.1. The new SMA model hypersensitive to RNAi makes it possible to identify suppressor and enhancer modifiers

Our aim is to perform an RNAi *by feeding* screening to identify SMA modifiers that play their role also in neurons and that can rescue (*suppressor modifiers*) or worsen (*enhancer modifiers*) the neurodegenerative phenotype caused by *smn-1* silencing. To test whether the new SMA model hypersensitive to RNAi in the whole body, including the nervous system, reflects this goal, and to identify a suppressor control gene and an enhancer control gene for the screening, I performed a pilot RNAi *by feeding* experiment. Therefore, genes that had been shown in previous studies to be suppressor or enhancer modifiers were silenced in this strain.

Following the RNAi *by feeding* protocol previously described, I silenced as possible *enhancer* genes: *lin-45*, the *C. elegans* ortholog of *B-RAF* that is known to rescue the neurodegenerative phenotypes when overexpressed ubiquitously or only in MNs (Hensel *et al.*, 2021); and *tcab-1*, the *C. elegans* ortholog of WDR79, that is known to rescue the locomotion defect when pan-neuronally expressed (Di Giorgio *et al.*, 2017). Thus both genes, in principle, after silencing should worsen the neurodegeneration. In addition, two unpublished new SMA modifiers, identified in Di Schiavi's lab, were silenced as possible suppressor genes. Previous unpublished experiments, have shown that these two genes suppress the neurodegeneration caused by *smn-1* silencing when their function is impaired (patent application to be compiled). After silencing, the neurodegeneration was analyzed (figure 8.2).

The analysis of neurodegeneration following the silencing of putative suppressor and enhancer of SMA, confirms our hypothesis that a less severe neurodegenerative phenotype in this new SMA model, optimized for RNAi *by feeding*, allows the identification of enhancer and suppressor genes. Indeed, compared to the empty vector, the RNAi negative control (i.e. animals in which no gene is silenced), silencing of both *lin-45* and *tcab-1* worsens the neurodegenerative defect caused by *smn-1* silencing, and the silencing of both Suppressor 1 and Suppressor 2 rescues the neurodegenerative defect caused by *smn-1* deficiency. The mean of visible MNs, indeed, decreases from 12.26 of the Empty vector to 11.54 after *lin-45* silencing ($p = 0.0119$) and to 10.83 post *tcab-1* silencing ($p < 0.0001$). Additionally, the mean of visible MNs after silencing Suppressor 1 and Suppressor 2 increases from 12.26 of the Empty vector to 14.98 and 14.83, respectively ($p < 0.0001$). Thus, I selected *tcab-1* as *enhancer control gene* and Suppressor 1 as *suppressor control gene*.

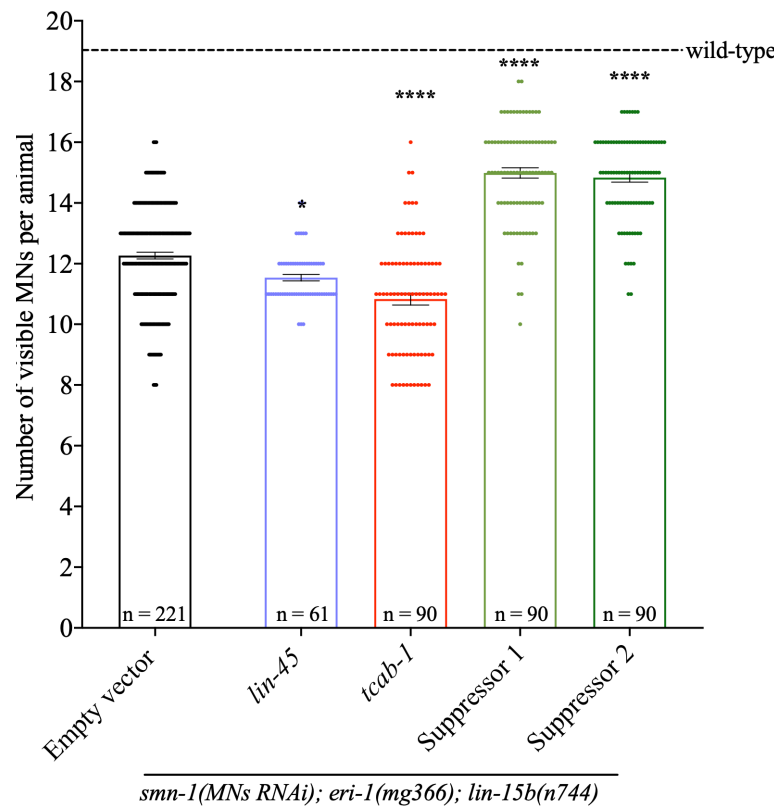


FIGURE 8.2. **SMA model hypersensitive to RNAi allows the identification of suppressor and enhancer genes:** the graph shows the number of visible MNs in the SMA model hypersensitive to RNAi after *lin-45* (violet bar), *tcab-1* (red bar), Suppressor 1 (light green bar), Suppressor 2 (dark green bar) gene silencing. Empty vector is the *negative control* of the silencing, and shows the number of visible MNs of animals when no gene is silenced *by feeding*. The motoneurons were identified as distinct mCherry-expressing cells and quantified for each animal. *lin-45* (* $p=0.0119$ versus Empty vector) and *tcab-1* ($p<0.0001$ versus Empty vector) worsen the neurodegeneration; Suppressor 1 ($p<0.0001$ versus Empty vector) and Suppressor 2 ($p<0.0001$ versus Empty vector) ameliorate it. The dashed line shows the number of viable MNs in wild-type animals (19). n is the total number of animals observed. Graphs are scatterplots with bars and whiskers displaying mean and SEM. Each dot represents the number of visible MNs counted per each animal tested. One-way ANOVA with Kruskal-Wallis non-parametric analysis and Dunn's multiple comparison test were performed.

8.1.2. The new SMA model hypersensitive to RNAi makes it possible to identify modifiers that act also in neurons

As mentioned above, the new SMA model hypersensitive to RNAi should allow the silencing of candidate genes in all cells, including neurons. I tested this property using the suppressor candidate control genes, Suppressor 1 and Suppressor 2. In particular, Suppressor 1 is known to have a muscular function, while Suppressor 2 is known to have a neuronal-specific function, being expressed only in the nervous system.

Therefore, to demonstrate whether it was possible to silence neuronal genes in the new RNAi hypersensitive SMA model (*smn-1(MNs RNAi); eri-1(mg366); lin-15b(n744)* animals), I adopted an indirect approach by silencing Suppressor 1 and Suppressor 2 in the SMA model non-sensitive to RNAi in neurons, so only *smn-1(MNs RNAi)* animals; then I scored the neurodegeneration (figure 8.3). Since in this strain neurons are resistant to interference, a neuronal gene should not be silenced.

The analysis of the number of visible MNs following silencing of Suppressor 1 and Suppressor 2 in the SMA model not hypersensitive to RNAi in all cells, showed that Suppressor 1, the non-neuronal modifier gene, confirmed the rescue of the neurodegenerative phenotype caused by *smn-1* silencing in MNs; on the contrary, Suppressor 2, the neuron-specific modifier gene, did not. The mean of number of visible MNs, indeed, increases from 11.6 in animals in which no gene is silenced (Empty

vector) to 15.61 after Suppressor 1 silencing, while it doesn't change when Suppressor 2 is silenced. On the other hand, the analysis of the number of visible MNs following Suppressor 1 and Suppressor 2 silencing in the SMA model hypersensitive to RNAi in all cells including neurons, confirmed that both suppressor genes partially rescued the neurodegenerative phenotype caused by *smn-1* deficiency. Indeed, the mean number of visible MNs increases from 11.08 in animals in which no gene is silenced (Empty vector) to 14.98 after Suppressor 1 silencing and to 14.83 after Suppressor 2 silencing.

These results demonstrate, in an indirect way, that the SMA model hypersensitive to RNAi in all cells including neurons indeed allows the silencing also of neuronal genes, such as Suppressor 2, confirming the validity of this new strain as a perfect model for carrying out RNAi by feeding screening to identify and select new SMA modifiers for further studies.

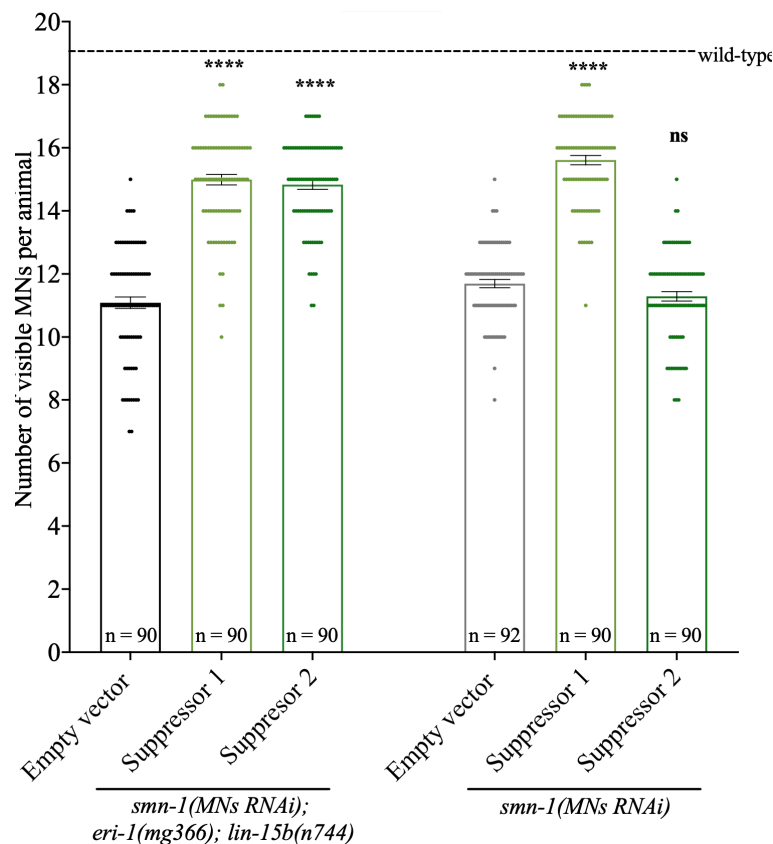


FIGURE 8.3. The SMA model hypersensitive to RNAi allows the identification of neuronal modifiers: the graph shows the number of visible MNs after Suppressor 1 (light green bar) and Suppressor 2 (dark green bar) gene silencing in both SMA model hypersensitive to RNAi (left) and SMA model non-hypersensitive to RNAi in neurons (right). Empty vector (black and grey bars) is the *negative control* of the silencing, and shows the number of visible MNs of animals in which no gene is silenced. The motoneurons were identified as distinct mCherry-expressing cells and quantified for each animal. The SMA model hypersensitive to RNAi allows the silencing also of neuronal genes, such as Suppressor 2 (**** $p < 0.0001$ versus Empty vector). Suppressor 2 RNAi, indeed, didn't affect the SMA model insensitive to RNAi in neurons ($p > 0.9999$ versus Empty vector). The dashed line shows the number of viable MNs in wild-type animals (19). n is the total number of animals observed. Graph are scatterplots with bars and whiskers displaying mean and SEM. Each dot represents the number of visible MNs counted per each animal tested. One-way ANOVA with Kruskal-Wallis non-parametric analysis and Dunn's multiple comparison test was performed.

8.2. Speeding up the screening

Time is a critical element in studying a disease and finding a cure or, as in the case of SMA, complementary treatments. It is therefore important to use models that allow to obtain rapid results. As described above, one of the main advantages of *C. elegans* as experimental model is its fast life

cycle, which allows large numbers of animals to be analyzed and specific biological questions to be answered in a relatively short period of time. This feature is crucial in genetic screenings to identify new putative candidate genes for the development of new therapeutic strategies and to better elucidate disease pathomechanisms, given that the same genetic approach can quickly select more than one candidate for further study. When setting up and fine-tuning a screening, one aspect to consider is the ability to select a faster analysis mode that also optimizes the workload as much as possible. In this regard, I carried out a pilot experiment to test two different ways of quantifying the number of visible MNs and the time taken to analyze them. The analysis method canonically used to quantify neurodegeneration in our lab includes anesthetizing worms, preparing slides and then using a compound microscope to count the number of visible MNs in each animal with high magnification. Using this strategy, it is possible to analyze 100 animals in 1 hour, starting from the preparation of the slides. To assess whether this strategy could be speeded up by analyzing worms directly in the plates and avoiding slides preparation, a stereo microscope fitted with a 2× zoom was tested.

8.2.1. Immobilization strategies to use a stereomicroscope

To assess whether the stereomicroscope could be used to speed up the neurodegeneration analysis, I first determined whether an anesthesia was required to stop worms in the plate. Therefore, I assessed the number of visible MNs in wild-type controls animals when awake; then animals were immobilized cooling down the plates on ice for different times (5', 10' and 20'); moreover for testing anesthesia I treated them with different amounts of 100% chloroform (10 μ L, 15 μ L and 20 μ L) for 15'. The anesthetization with chloroform was achieved by attaching a coverslip to the inner of the plates lid, pipetting onto it the amount of chloroform needed and then closing the plate.

Table 8.1 lists all the conditions tested, their effect on the animals and whether neurodegeneration was assayed.

Anesthetization strategy	Effect on animals	Neurodegeneration tested
None	Awake worms	Yes
Maintaining plates 5' on ice	Awake worms after few minutes	Yes
Maintaining plates 10' on ice	Awake worms after few minutes	Yes
Maintaining plates 20' on ice	Anesthetized animals	Yes
Treating worms with 10 μ L chloroform 15'	Awake worms	No
Treating worms with 15 μ L chloroform 15'	Anesthetized animals	Yes
Treat worms with 20 μ L chloroform 15'	Dead worms	No

TABLE 8.1. **Immobilization strategies and their effect:** the table shows the strategies tested to anesthetize worms and their effect on animals.

Among the strategies tested, treating the worms with the lower amount of chloroform didn't stop the worms, possibly because this amount of compound was too low; whereas treating the worms with the highest amount of chloroform killed the animals; these two conditions were thus excluded. The number of visible MNs per animal was then counted in the other conditions and compared with the number of visible MNs counted using slides and a compound microscope (figure 8.4).

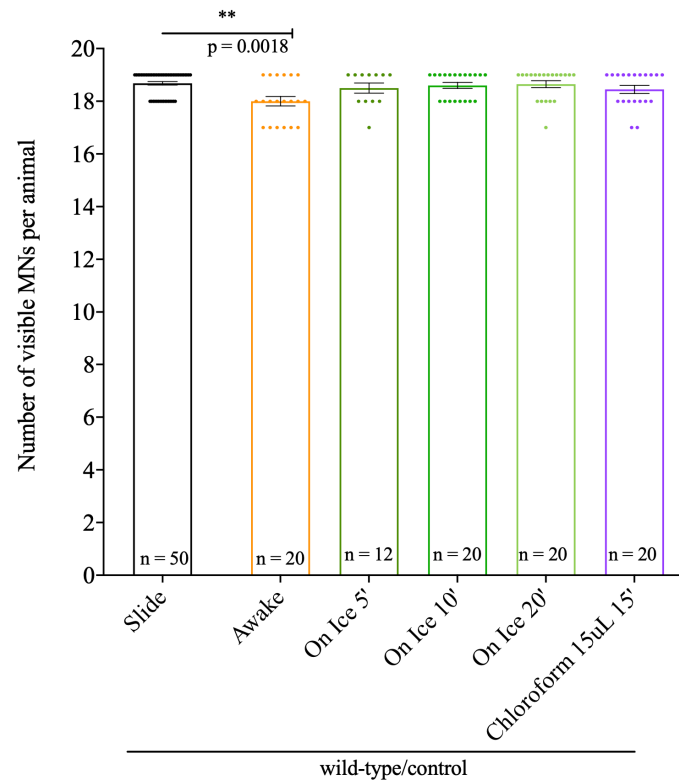


FIGURE 8.4. Immobilization strategies and their effect on neurodegeneration: the graph shows the number of visible MNs in wild-type animals analyzed on slides (black bars), in awake worms (orange bar), in animals immobilized maintaining the plates for 5' on ice (dark green bar), for 10' on ice (green bar), for 20' on ice (light green bar), animals anesthetized using 15 μ L chloroform for 15' (purple bar). The motoneurons were identified as distinct GFP-expressing cells and quantified for each animal. Graphs are scatterplots with bars and whiskers displaying mean and SEM. Each dot represents the number of visible MNs counted per each animal tested. n is the number of analyzed animals. One-way ANOVA with Kruskal-Wallis non-parametric analysis and Dunn's multiple comparison test were performed.

First, the analysis of neurodegeneration showed that it is possible by using a stereomicroscope to count the number of visible MNs, thus avoiding slide preparation and the need for a compound microscope. Although it is possible to assess neurodegeneration in awake animals, the moving worms don't allow a proper evaluation of this phenotype since it is often hard to visualize the MNs in the vulva area. The mean of visible MNs in awoken animals is indeed 18 over the expected 19 ($p=0.0018$). Then, regarding the immobilization strategies, the most effective ones were maintaining plates 20' on ice and treating animals with 15 μ L chloroform for 15'. In addition, while it's possible to quantify the neurodegenerative effects by keeping the plates on ice for 5' or 10', since the analysis is consistent with the one performed using the compound microscope, neither of these timing is sufficient to immobilize the animals for longer, as they wake up after few minutes, making hard the whole analysis.

Thus, the more efficient methods of immobilizing the animals were keeping the plates on ice for 20' and treating the worms with 15 μ L chloroform for 15'; these two strategies were selected for further testing and used to analyze the number of visible MNs in the SMA model in order to confirm their efficacy (figure 8.5).

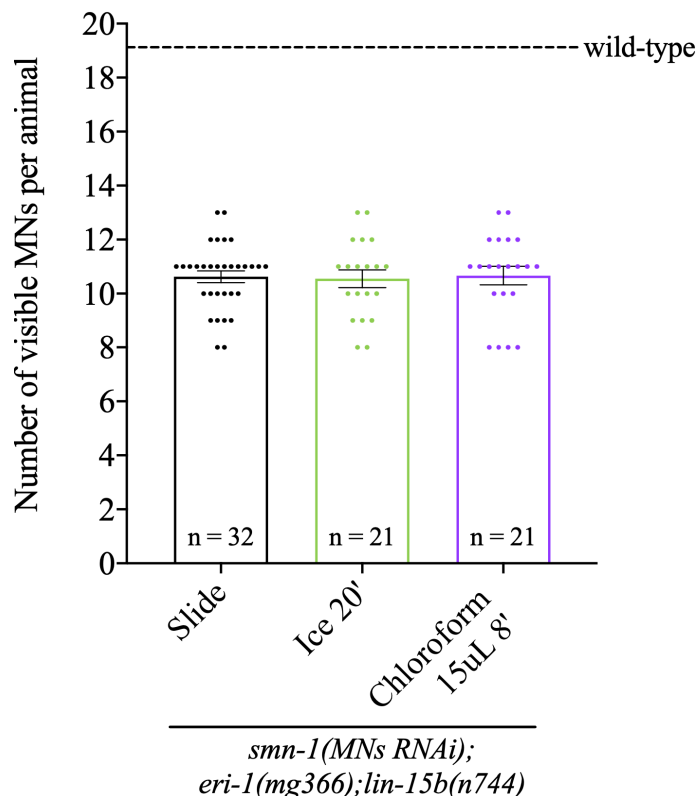


FIGURE 8.5. **Anesthetization strategies and their effect on SMA model:** the graph shows the number of visible MNs in the SMA model hypersensitive to RNAi in all cells analyzed on slides (black bars), immobilized by maintaining plates for 20' on ice (light green bar) and anesthetized using 15 μ L chloroform for 15' (purple bar). The motoneurons were identified as distinct mCherry-expressing cells and quantified for each animal. The dashed line shows the number of viable MNs in wild-type animals (19). Graph is scatterplots with bars and whiskers displaying mean and SEM. Each dot represents the number of visible MNs counted per each animal tested. n is the number of analyzed animals. One-way ANOVA with Kruskal-Wallis non-parametric analysis and Dunn's multiple comparison test were performed. All values are not statistically different.

The analysis of neurodegeneration confirmed the efficacy of both methods for immobilizing worms and checking neurodegeneration; in fact, the mean of the visible MNs in all the conditions tested was 10.6 and was not statistically different from each other. However, due to the chemical hazard of using chloroform, I chose ice as alternative method to try to speed up the analysis process.

8.2.2. Choosing the most efficient method to analyze neurodegeneration

As shown in the previous paragraph (8.2.1), counting the number of visible MNs using the fluorescence stereomicroscope, with the worms immobilized by keeping the plates on ice for 20', provide a viable alternative method to using the compound microscope and preparing slides. To assess whether this new alternative method could significantly speed up the screening analysis process and thus be adopted as the preferred method for the RNAi *by feeding* screening, I performed an RNAi *by feeding* test on the previously analyzed control genes. In particular, I

simultaneously performed two different analyses using both methods for quantifying the number of visible MNs. To determine which method was more efficient, the timing of the analysis process was counted starting from anesthetized worms. The results of the analysis are reported in figure 8.6.

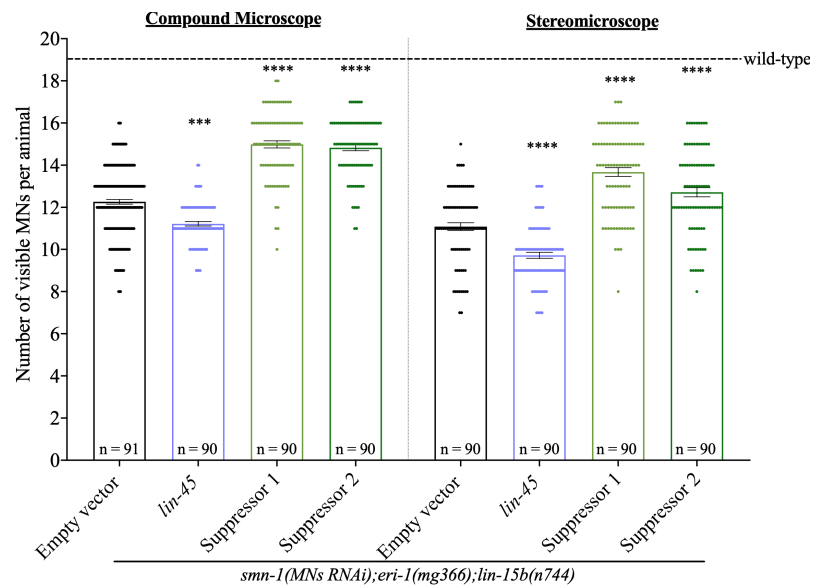


FIGURE 8.6. **Speeding up the screening:** the graph shows the number of visible MNs of SMA model hypersensitive to RNAi in all cells, post *lin-45* (violet bar), Suppressor 1 (light green bar) and Suppressor 2 (dark green bar) gene silencing. Empty vector is the *negative control* of the silencing, and shows the number of visible MNs of animals in which no gene is silenced. On the left side is shown the analysis performed using the compound microscope and preparing slides; on the right side is represented the analysis performed using the stereomicroscope and ice to anesthetize worms. The analysis is consistent with the previous ones, confirming both the *enhancer* role of *lin-45* and Suppressor 1 and Suppressor 2 *suppressors* role, and the efficiency of both methods to analyze neurodegeneration as well. The motoneurons were identified as distinct mCherry-expressing cells and quantified for each animal. The dashed line shows the number of visible MNs in wild-type animals (19). Graphs are scatterplots with bars and whiskers displaying mean and SEM. Each dot represents the number of visible MNs counted per each animal tested. n is the number of analyzed animals. One-way ANOVA with Kruskal-Wallis non-parametric analysis and Dunn's multiple comparison test were performed: *** (p= 0.0001 vs Empty vector), **** (p<0.0001 vs Empty vector).

The analysis of the neurodegeneration confirms previous results (figure 8.2), thus the new SMA model allows to identify both SMA *enhancer* and *suppressors*. Moreover, it also confirms that both methods tested for analysis are feasible to efficiently perform the screening and identify *enhancer* and *suppressor* genes. However, the chronometric analysis showed that the analysis carried out with the stereomicroscope was 2h long, starting from animals' immobilization, suggesting that the analysis of each gene should be 30' long. The analysis using the compound microscope was 2h20' long, starting from animal anesthetization; this suggests that the analysis of each gene should be 36' long. Although it's faster to analyze neurodegeneration using a dissecting microscope, the time saved is not enough to significantly justify a change in methodology to speed up the screening. So, although this new method could be useful for quantifying this phenotype, it's not enough advantageous to be used for the whole screening.

CHAPTER 9

An RNAi *by feeding* screening to identify new SMA *modifiers*

To address the devastating effects of SMA, innovative approaches are needed to elucidate the mechanisms underlying the disease, identify therapeutic targets and develop new therapeutic strategies. As mentioned in the previous chapters, network biology has been widely shown to be a promising tool to achieve all these goals and to make a significant contribution in the SMA field.

This is supported by the phospho-array and network biology approach developed by Prof. Claus' group (Hensel *et al.* 2021), which allowed the identification and characterization of B-RAF role in neurodegeneration as a new SMA modifier (Hensel *et al.*, 2021) (see Chapter 1 paragraphs 1.5, 3.4 and Chapter 7). Besides *B-RAF/lin-45*, the phospho-array approach developed by our collaborators, allowed the identification of other 37 dysregulated proteins in Spinal Muscular Atrophy, whose role in the disease needs to be confirmed and characterized. In this regard, because of its established role as a perfect complementary model organism for the identification of new SMA modifiers, I chose *C. elegans* for an RNAi *by feeding* screening of the entire network to identify and select the most interesting dysregulated proteins for further studies. Analyzing 37 candidate modifiers by genetic screening as modifiers of neuron survival in any other setting and animal model would have been impossible.

As described above, I tested a new *C. elegans* SMA model to optimize the screening efficiency and identify modifiers that can play their role in all the tissues, including the nervous system. This chapter focuses on the RNAi *by feeding* screening and describes the experiment performed to select the top candidate genes for further investigation.

9.1. Retrieve information about candidate genes

One of the advantages of using *C. elegans* as a model organism to study human diseases is the good homology of its genome to the human one. Therefore, before performing the RNAi *by feeding* screening, I performed a bioinformatics analysis using several tools (Ortholist 2.0, TreeFam and NCBI BLAST/BLAT), to identify the *C. elegans* homologs of the 37 proteins of the network. Unfortunately, according to the bioinformatic tools adopted, three out of thirty-seven of the putative new SMA modifiers from Hensel *et al.*, 2021 (i.e. MED1, IRS1 and IKK γ) have no homologs in *C. elegans* and were not tested.

First, I used Ortholist 2.0 (Kim *et al.*, 2018). This is a compendium of *C. elegans* genes with human homologs, based on a meta-analysis strategy that compiles different orthology-prediction programs (Kim *et al.*, 2018). The analysis was set up by selecting the maximum number of orthology-prediction programs available, and 83 *C. elegans* homologs of the candidates genes were identified. Then I performed a NCBI BLAST/BLAT alignment and TreeFam - database of animal gene trees

analysis to confirm the Ortholist 2.0 results. Of course, these 83 *C. elegans* genes are not all orthologs to the 37 genes, but since the screening in *C. elegans* allows to silence a high number of genes in a rapid time, we preferred to start with a larger cohort adding no bias to the selection. Then, I analyzed all these candidate homologs and their human counterparts to gather information on their expression, biological functions, interactions, as well as, their previously known role in SMA or in neurodegeneration. Furthermore, focusing on the *C. elegans* candidates, I also collected from wormbase.org information regarding the feasibility of RNAi *by feeding* and any known deleterious phenotype related to RNAi. With this approach, I found out that, of the 83 *C. elegans* homologs, 7 were not testable *via* RNAi *by feeding* because they were not present in the RNAi *by feeding* library adopted for the analysis and available in the lab. Of the 76 testable and remaining genes, reported in black in table 9.1 (table 9.1omitted), 14 were essential genes, thus causing lethality when constitutively silenced, hence not testable for neurodegeneration at young adult stage.

Table 9.1 omitted

TABLE 9.1. **Candidates genes found in *C. elegans*:** the table above shows the human candidate genes and their *C. elegans* homologs tested through RNAi *by feeding*. The *C. elegans* genes in red are essential ones, the *C. elegans* genes in bold are homologs to several human genes in the table. The *C. elegans* genes in red and underlined are essential genes lethal when cell-specifically silenced.

9.2. Identification of new target genes involved in SMA

As previously described, of the 83 *C. elegans* candidate genes, 76 cannot be tested *via* RNAi *by feeding*, and 14 were essential genes. To confirm the phospho-array results *in vivo*, to elucidate whether the candidate genes play a role in SMA-related neurodegeneration and clarify whether they act as *enhancers* or *suppressors* of neurodegeneration, at first, I silenced the 62 remaining non-essential genes testable by RNAi *by feeding* in the new SMA model hypersensitive to RNAi in all cells, including neurons. Following this strategy, it is possible to identify new putative *modifiers* capable of rescuing or worsening neurodegeneration, that may play their role in nervous system and/or in other tissues. Thus, the 62 candidate genes were silenced in the new SMA model, following the RNAi *by feeding* protocol, and then neurodegeneration was analyzed after two generations of silencing. An empty vector was used as a negative control for silencing, while *tcab-1* and Suppressor 1 were the *enhancer* and *suppressor* control genes, respectively.

The analysis of the neurodegeneration shows that among the 62 candidate genes tested, 35 are new SMA *modifiers* since they act as neurodegeneration *enhancers* or *suppressors*. Several different experiments were performed during time, and in figure 9.1 it is shown the pooling of all of them.

The analysis of the neurodegeneration revealed that among the 62 candidate genes tested, 35 were *modifiers* of the neurodegeneration, since they were able to significantly rescue or worsen this phenotype. Particularly, 13 candidate genes are *suppressor genes* and 21 are *enhancer genes*. In addition to the new candidate genes, also *lin-45* was silenced and confirmed as SMA *enhancer enhancer*. *lin-45* is the *B-RAF* *C. elegans* ortholog, and this new result further confirms *lin-45* role in neuroprotection since its silencing worsened the neurodegeneration, suggesting an important role in neuron survival.

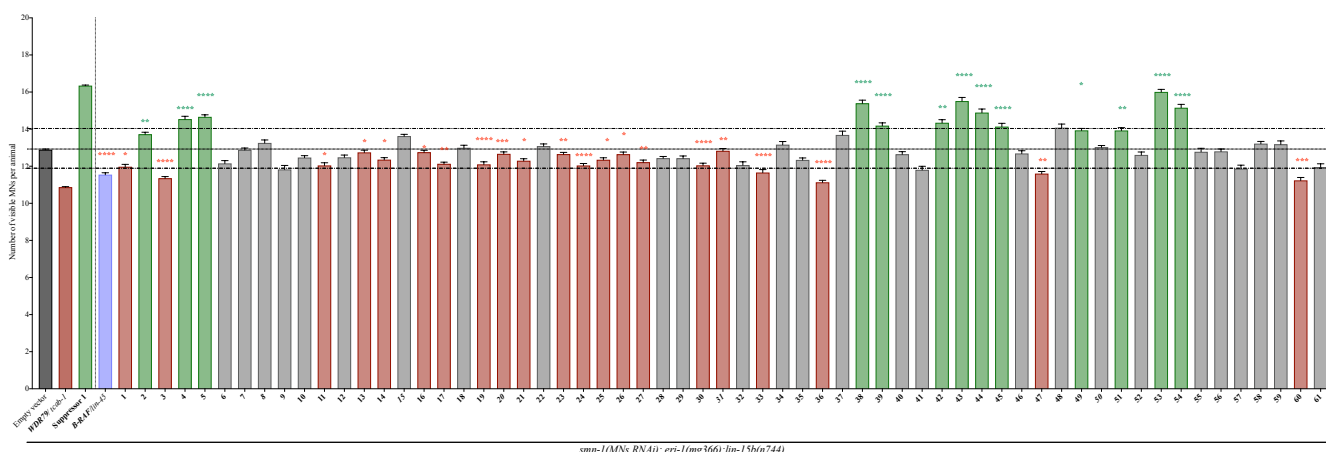


FIGURE 9.1. Identification of new modifiers of SMA neurodegeneration: the figure represents the pool of all 17 experiments performed to silence the 62 candidate genes plus *lin-45*. The graph shows the number of visible MNs in the SMA model hypersensitive to RNAi in all cells, after silencing of *tcab-1* as enhancer control gene (dark red bar), Suppressor 1 as suppressor control gene (dark green bar), *lin-45* (violet bar), and all the 62 candidate genes. Empty vector is the *negative control* of the silencing, and shows the number of visible MNs of animals in which no gene is silenced. The red bars are *enhancers*, thus genes that when silenced exacerbate the neurodegeneration caused by *smn-1(MNsRNAi)*; the green bars are *suppressors*, thus genes that when silenced rescue the neurodegeneration caused by *smn-1(MNsRNAi)*; the grey bars are genes that when silenced do not affect the neurodegeneration caused by *smn-1* silencing. The motoneurons were identified as distinct mCherry-expressing cells and quantified for each animal. The thin dashed line represents the mean of visible MNs of the empty vector (13), while the two bold dashed lines below and above the thin one correspond to a difference of ± 1 MN from the control. In a wild type 19 neurons are visible (not shown). Bars above the upper bold dashed lines are genes that rescue the neurodegeneration of more than 1 MN, bars below the lower bold dashed lines are genes that worsen the neurodegeneration of more than 1 MN. The number of analyzed animal was >90 for each condition. The statistical analysis reported was performed in each experiment against the related control performed in parallel each time. One-way ANOVA with Kruskal-Wallis non-parametric analysis and Dunn's multiple comparison analysis were performed: **** (p<0.0001 versus Empty vector); *** (p=0.0001 versus Empty vector); ** (p<0.001 versus Empty vector); * (p<0.01 versus Empty vector).

As mentioned above to perform the entire screening and silence all the candidate genes, I performed different experiments. Therefore, the graph in figure 9.1 is just a representation of the overall result and includes the statistical analysis against EV (Empty vector) performed in each individual experiment.

A clearer way to show these results including statistical analysis is a volcano plot (figure 9.2), that represents the variation of visible MNs between the SMA animal model with no gene silenced (Empty vector of each single experiment) and animals after the candidate genes silencing (figure 9.2 x axis), related to the *p* value obtained after each single experiment (figure 9.2 y axis). Higher is the *p* value (the value on the y axis of the volcano plot) higher is the statistical difference between the gene tested and its control (Empty vector).

Thus, the silencing of the candidate genes in the new SMA model hypersensitive to RNAi in all cells including neurons allowed the identification of new 34 modifiers of the neurodegeneration and further confirms *B-RAF/lin-45* neuroprotective role. These 35 candidates genes were selected as *positive hits* for further studies in order to better elucidate their role in neurodegeneration.

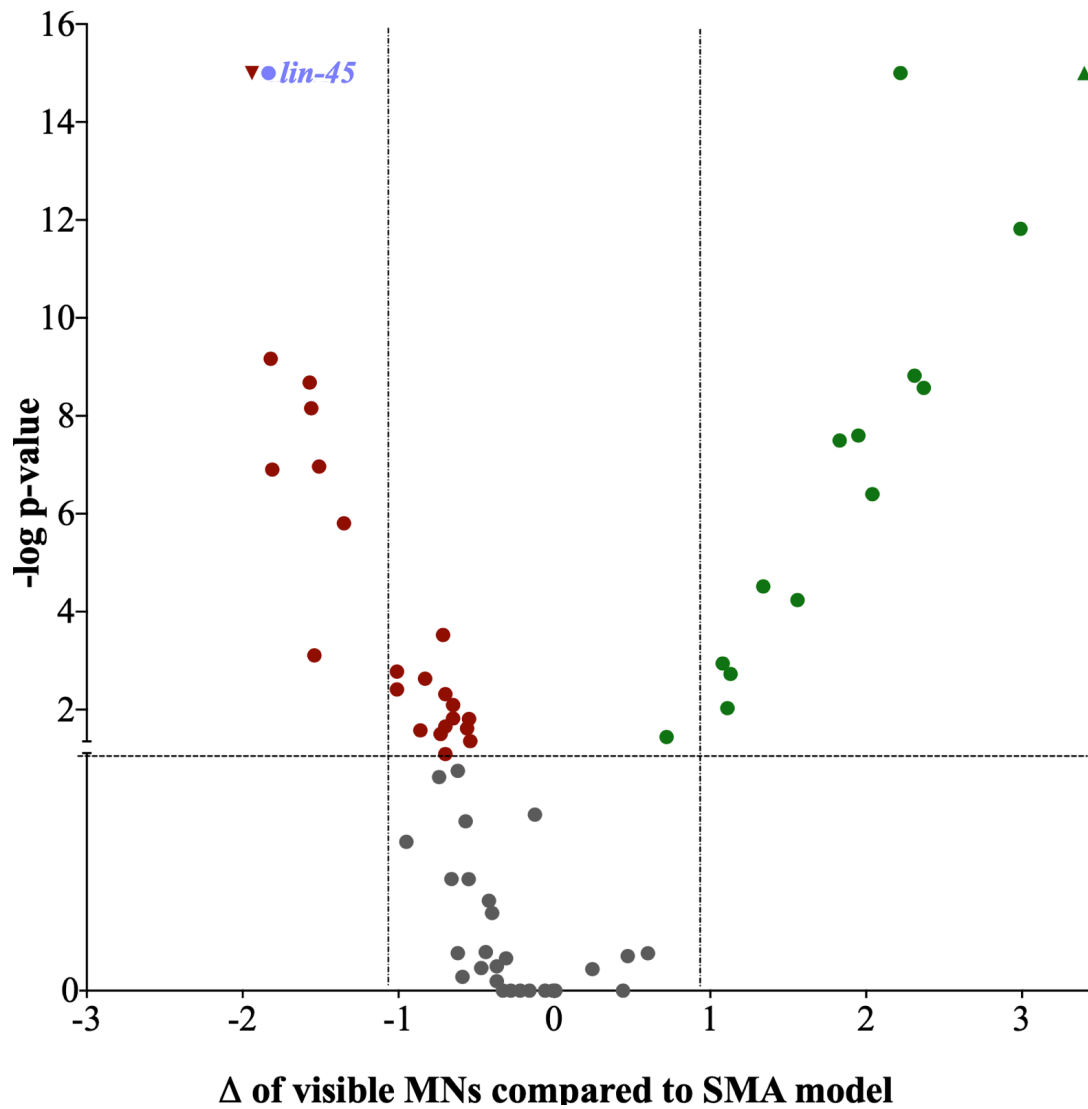


FIGURE 9.2. **Volcano plot to represent the new SMA *modifiers* identified in the screening:** the graph shows the result of the screening following the silencing of the 62 candidate genes using the SMA model hypersensitive to RNAi in all cells. The x axis indicates the difference in visible MNs between the SMA model (empty vector of each single experiment) and the gene tested; 0 corresponds to the number of visible MNs in the SMA model (approximately 13 MNs). On the y axis is reported the p-value for each gene related to its own empty vector (One-way ANOVA with Kruskal-Wallis non-parametric analysis and Dunn's multiple comparison analysis). The vertical dashed lines indicate a Δ of visible MNs compared to SMA model equal to minus 1 neuron (left line) or plus 1 neuron (left line). The grey dots between the dashed line are the genes that if silenced do not cause any significant change in the neurodegenerative defect. The green dots on the right are the *modifiers* that if silenced suppress the neurodegeneration caused by *smn-1* silencing, while the red dots on the left are the *modifiers* that if silenced worsen the neurodegeneration and that are neuroprotective. The violet dot represents *lin-45* and the triangles are the control genes.

9.3. Identification of target genes involved in general neurodegeneration

As mentioned, an enhancer gene is a gene that exacerbates the neurodegeneration caused by *smn-1* silencing. A gene capable of worsening the neurodegeneration when silenced, can also be a neuroprotective gene, because it is crucial for neurons survival when present. Since the enhancer candidate genes were silenced in *smn-1(MNs RNAi)* genetic background, the previous experiment (see paragraph 9.2) isn't sufficient to determine whether an enhancer gene has also a general neuroprotective role or the neuroprotective role is specifically related to *smn-1* deficiency.

Therefore, to further characterize the candidate enhancer genes and to determine whether or not their neuroprotective effect was dependent on *smn-1* deficiency, I silenced the 21 *enhancers* genes and *BRAF/lin-45* in a strain hypersensitive to RNAi in all cells including neurons, but wild-type for *smn-1*. Using this genetic background, a gene involved in neuroprotection when silenced should cause itself a neurodegeneration; instead, a gene involved in neuroprotection only when *smn-1* levels are reduced will not cause any neurodegeneration when silenced. The empty vector, was used as negative control for silencing; *tcab-1* was the neuroprotective control gene (figure 9.3).

By silencing the enhancer candidate genes in wild-type background we were able to identify for the first time 13 genes involved in neuroprotection independently from *smn-1* expression levels. Their silencing in the strain hypersensitive to RNAi in all cells including neurons and wild-type for *smn-1*, indeed, causes a statistically significant reduction of the number of visible MNs suggesting that their general crucial role in neuron survival is not directly related to *smn-1* deficiency. To further confirm these results and better elucidate these 13 candidates' function in neurons further experiments are required, also to exclude a possible synergistic role with *smn-1*.

Of the 22 enhancer gene silenced in wild-type background, 9 of them do not affect the number of visible MNs suggesting that their neuroprotective effect is strongly related to *smn-1* deficiency. Interestingly, *lin-45* silencing in wild-type background (figure 9.3) didn't cause any neurodegeneration, suggesting that the neuroprotective effect of this gene, previously characterized, is *smn-1* dependent.

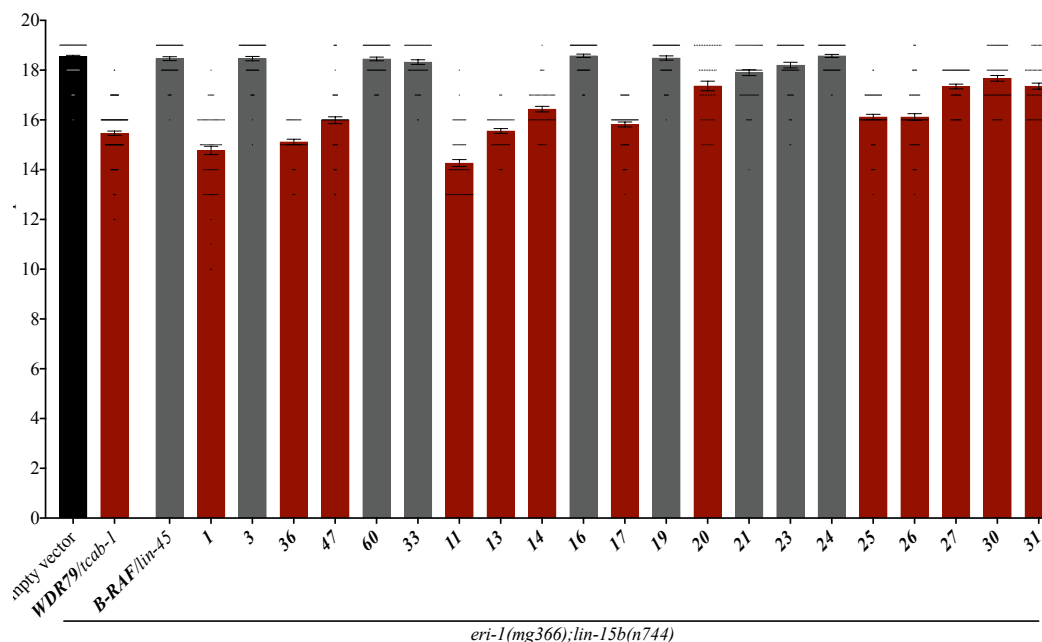


FIGURE 9.3. Identification of 13 new neuroprotective genes: the figure represents the pool of 5 experiments performed to silence the 21 enhancer genes and *lin-45*. The graph shows the number of visible MNs of animals hypersensitive to RNAi in all cells, after *tcab-1* (enhancer control gene, first red bar) silencing, or after silencing all the 21 enhancer genes and *lin-45*. Empty vector is the *negative control* of the silencing, and shows the number of visible MNs of animals in which no gene is silenced. The red bars are *enhancers* with a general neuroprotective role since they cause neurodegeneration when silenced also in wild-type background ($p < 0.001$ versus Empty vector); the grey bars are *enhancers* involved in neuroprotection only when *smn-1* is silenced, since their silencing in wild-type background does not affect the number of visible MNs ($p > 0.05$ versus Empty vector). The motoneurons were identified as distinct mCherry-expressing cells and quantified for each animal. Graphs are scatterplots with bars and whiskers displaying mean and SEM. Each dot represents the number of visible MNs counted per each animal tested. The number of analyzed animal was >50 for each condition. The statistical analysis reported was performed for each experiment with the related control performed in parallel. One-way ANOVA with Kruskal-Wallis non-parametric analysis and Dunn's multiple comparison test were performed.

9.4. Identification of neuron-specific modifiers

Although spinal muscular atrophy has neurological features that classify it as a neurodegenerative disease, there is an increasing evidence of its multi-system nature (Yeo and Darras, 2020). Therefore, given a pool of candidate genes that may be involved in the disease, it may be pivotal to determine whether their neuroprotective or neurodegenerative role is specifically played in neurons or whether other tissues are also involved. Thus, I employed an indirect approach by silencing the 35 *positive hits* in the SMA model insensitive to RNAi in neurons (see Chapter 8 paragraph 8.1.2). Following this method, an *enhancer gene* or a *suppressor gene* with a neuron-specific role, will not show any effect in this genetic background, since neurons are refractory to interference and neuron-specific genes are not affected by silencing. On the other hand, an enhancer or suppressor gene that has a non-neuronal role, or acts not only in neurons but also in other tissues, can be efficiently silenced and will affect the number of visible MNs.

9.4.1. Two enhancer genes showing a general neuroprotective effect, play their role in neurons

To further investigate the 13 enhancers with a general neuroprotective effect (red bars in figure 9.3), thus also visible in wild type background, firstly we silenced in the SMA model insensitive to RNAi in neurons (figure 9.4).

The analysis of the neurodegeneration shows that among the 13 *positive hits* with a general neuroprotective role, only two, (7 words omitted), play their role in neurons; in fact they do not affect the neurodegeneration caused by *smn-1* silencing ($p > 0.3$ vs Empty vector). The strain used is insensitive to RNAi in neurons and a neuronal gene cannot be silenced since this class of cells is resistant to the interference. The remaining 11 genes tested retained their neuroprotective role even in this genetic background, suggesting that their effect could be exerted both in the nervous system and in other tissues, synergistically, or only in a tissue other than the nervous system.

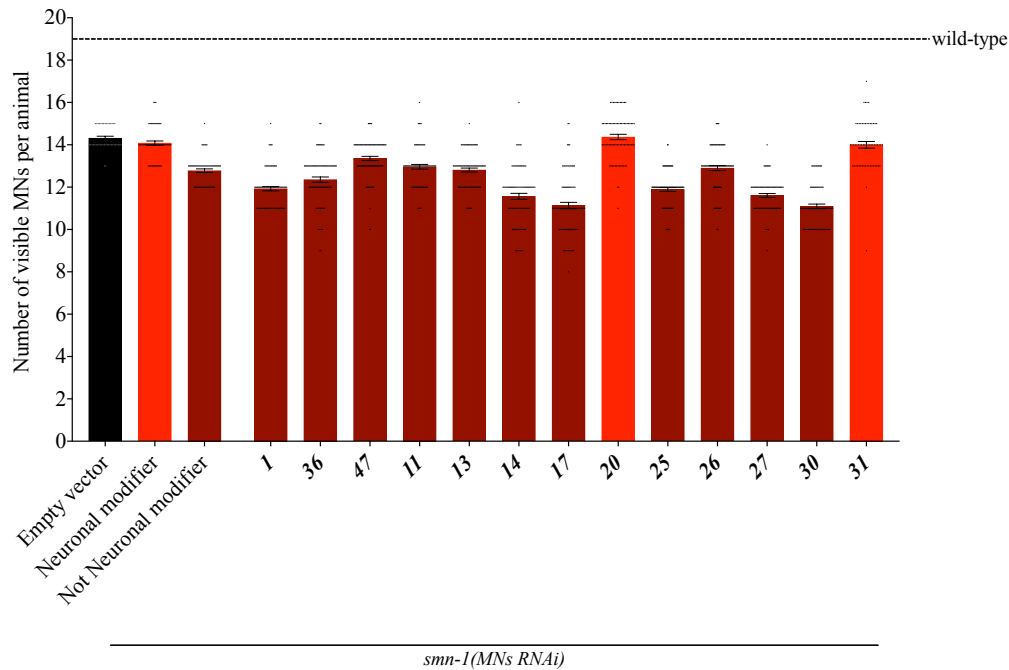


FIGURE 9.4. Two neuroprotective genes play their role in neurons: the figure represents the pool of 6 experiments performed to silence the 13 enhancer genes with general neuroprotective role. The graph shows the number of visible MNs of SMA animals insensitive to RNAi in neurons after silencing of the candidate genes with a general neuroprotective effect. Empty vector (black bar) is the *negative control* of the silencing, and shows the number of visible MNs of animals in which no gene is silenced. The first red bar (Neuronal modifier) shows the mean of visible MNs post-silencing of a neuronal control gene; the first dark red bar (non-Neuronal modifier) shows the mean of visible MNs post-silencing of a non-neuronal control gene. A neuronal gene can't be efficiently silenced in this strain; thus, it doesn't affect the neurodegeneration. The dark-red bars are *enhancer* modifiers with a general neuroprotective effect, not playing their role in neurons since they exacerbate neurodegeneration when silenced ($p < 0.001$ vs Empty vector); the two light red bars are *enhancer* modifiers playing their role in neurons since they do not affect the neurodegeneration caused by *smn-1* silencing ($p > 0.3$ vs Empty vector). The motoneurons were identified as distinct mCherry-expressing cells and quantified for each animal. The dashed line shows the number of visible MNs in wild-type animals (19). Graphs are scatterplots with bars and whiskers displaying mean and SEM. Each dot represents the number of visible MNs counted per each animal tested. The number of analyzed animal was >50 in each condition. The statistical analysis reported was performed for each experiment with the related control performed in parallel. One-way ANOVA with Kruskal-Wallis non-parametric analysis and Dunn's multiple comparison test were performed.

9.4.2. Four enhancer genes, which neuroprotective effect is related to *smn-1* deficiency, play their role in neurons

Following the previously described strategy, I silenced in the SMA model insensitive to RNAi in neurons the 9 *enhancer gene*, whose neuroprotective effect occur only in *smn-1(MNs RNAi)* genetic background, thus that their neuroprotective effect is strongly related to *smn-1* deficiency. Then I evaluated if their neuroprotective effect was conserved also in this new genetic background (figure 9.5).

The analysis of the neurodegeneration showed that among the 9 *enhancer* genes silenced in the SMA model insensitive to RNAi in neurons, five affect neurodegeneration also in this genetic background, suggesting that their role is played beyond neurons. The remaining four enhancer genes do not affect the neurodegeneration in this genetic background, suggesting their neuron-specific role. In support of this result come the data regarding *B-RAF/lin-45* that was previously demonstrated to exert a neuronal-specific neuroprotective role (Hensel *et al.*, 2021), 21 words omitted.

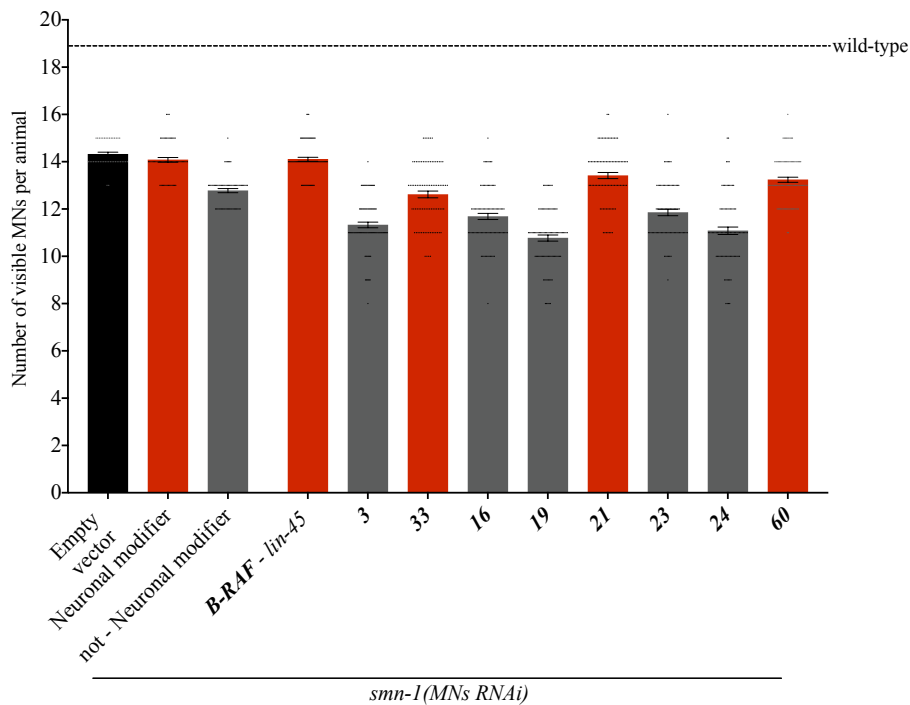


FIGURE 9.5. **Four enhancer genes play their role in neurons:** the figure represents the pool of 6 experiments performed to silence the 9 enhancer genes with neuroprotective role related to *smn-1* deficiency. The graph shows the number of visible MNs of SMA animals insensitive to RNAi in neurons after silencing the *enhancer* genes with neuroprotective effect related to *smn-1* deficiency. Empty vector (black bar) is the *negative control* of the silencing, and shows the number of visible MNs of animals in which no gene is silenced. The first red bar (Neuronal modifier) shows the mean of visible MNs post-silencing of a neuronal control gene; the first grey bar (non-Neuronal modifier) shows the mean of visible MNs post-silencing of a non-neuronal control gene. A neuronal gene can't be silenced in this strain; thus, it doesn't affect the neurodegeneration. The grey bars are *enhancer* modifiers whose neuroprotective effect occur in cells different from neurons since they exacerbate neurodegeneration when silenced in this genetic background ($p < 0.001$ vs Empty vector); the four red bars are *enhancer* modifiers playing their role in neurons since they do not affect the neurodegeneration caused by *smn-1* silencing ($p > 0.3$ vs Empty vector). The motoneurons were identified as distinct mCherry-expressing cells and quantified for each animal. The dashed line shows the number of visible MNs in wild-type animals (19). Graphs are scatterplots with bars and whiskers displaying mean and SEM. Each dot represents the number of visible MNs counted per each animal tested. The number of analyzed animal was > 50 for each condition. The statistical analysis reported was performed for each experiment with the related control performed in parallel. One-way ANOVA with Kruskal-Wallis non-parametric analysis and Dunn's multiple comparison test were performed.

9.4.3. Nine suppressor genes play their role in neurons

As mentioned above, a suppressor gene is a gene capable of ameliorating the neurodegeneration caused by *smn-1* knockdown. Following the same methods used to better characterize the enhancers effect, the 13 suppressor genes selected after the screening in the SMA model hypersensitive to RNAi in all cells including neurons, were also silenced in the different SMA model, which is insensitive to RNAi in neurons, in order to define whether their suppressor effect is exerted in neurons or also in other tissues (figure 9.6).

The results of the analysis showed that of the 13 *suppressor* genes silenced in the SMA model insensitive to RNAi in neurons, 4 are suppressor genes playing their role beyond neurons since their silencing affect the number of visible MNs also in this new genetic background; and 9 are neuronal-specific genes since they didn't affect the neurodegeneration, suggesting that they are not silenced in this strain.

To further support the consistency of these results, it is known that the non-neuronal candidate gene, *54* is expressed also in muscles, while the neuronal suppressor *51* has a neuronal function **11 words omitted**. This set of experiments allows to distinguish the neuronal *modifiers* from those that play their role beyond the neurons. However, regarding the non-neuronal candidate genes, due to the properties of the SMA model employed, I wasn't able to precisely define either the tissue in which

they play their role or whether their enhancer or suppressor action is synergistic in the nervous system or exclusively non-neuronal. To understand this aspect we will perform further experiments.

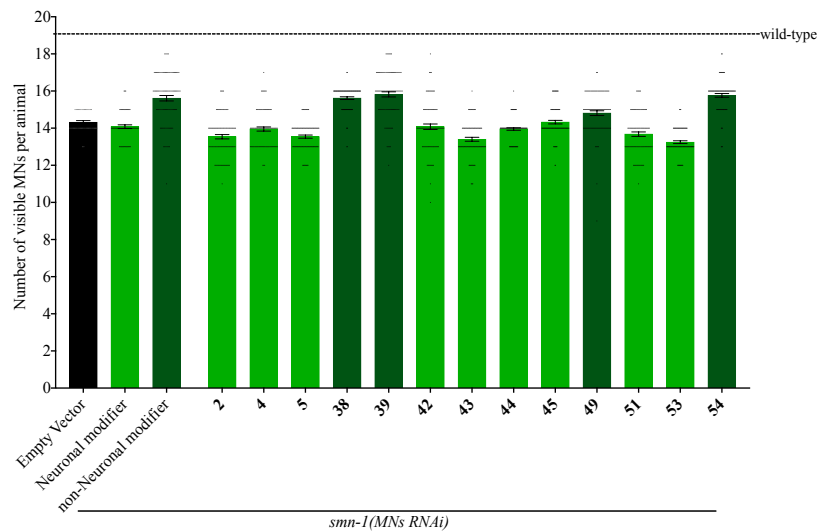


FIGURE 9.6. Four suppressor genes play their role in neurons: the figure represents the pool of 6 experiments performed to silence the 13 suppressor genes. The graph shows the number of visible MNs of SMA animals insensitive to RNAi in neurons post silencing of the *suppressor* genes. Empty vector (black bar) is the *negative control* of the silencing, and shows the number of visible MNs of animals in which no gene is silenced. The first green bar (Neuronal modifier) shows the mean of visible MNs post-silencing of a neuronal control gene, the first dark green bar (non-Neuronal modifier) shows the mean of visible MNs post-silencing of a non-neuronal control gene. A neuronal gene can't be silenced in this strain; thus it doesn't affect the neurodegeneration. The dark green bars are *suppressor* modifiers whose neuroprotective effect occur in cells different from neurons since they ameliorate neurodegeneration when silenced in this genetic background ($p < 0.001$ vs Empty vector); the nine green bars are *suppressor* modifiers playing their role in neurons since they do not affect the neurodegeneration caused by *smn-1* silencing ($p > 0.1$ vs Empty vector). The motoneurons were identified as distinct mCherry-expressing cells and quantified for each animal. The dashed line shows the number of visible MNs in wild-type animals (19). Graphs are scatterplots with bars and whiskers displaying mean and SEM. Each dot represents the number of visible MNs counted per each animal tested. The number of analyzed animal was >50 for each condition. The statistical analysis reported was performed for each experiment with the related control performed in parallel. One-way ANOVA with Kruskal-Wallis non-parametric analysis and Dunn's multiple comparison test were performed.

9.5. A cell-specific RNAi by feeding approach to identify MNs-specific modifiers

There is an increasing evidence of the multi-system nature of spinal muscular atrophy (Yeo and Darras, 2020), but the disease primarily affects MNs, so the interest in identifying SMA modifiers that act specifically on this class of neurons remains critical. In this regard, the approach I previously adopted (see paragraph 9.4), allowed the identification of 15 *candidate genes* with a neuron-specific role in the neurodegeneration. In order to determine which of the neuronal modifier play its role specifically in motoneurons, I silenced them specifically in MNs using a neuron-specific RNAi by feeding approach. This strategy was carried out using a SMA *C. elegans* model optimized for cell-specific RNAi in MNs. This SMA strain carries: *eri-1(mg366);lin-15b(n744)* mutations that hyper-sensitized all the cells, including neurons, to RNAi; *rde-1(ne219)*, a loss of function mutation that causes systemic RNAi resistance; a transgene that specifically rescues in MNs *rde-1* and *sid-1*, a gene that promotes dsRNA spreading to neurons (Firnhaber and Hammarlund, 2013). Thus, these animals are resistant to RNAi in the whole body except for MNs.

In this strain the non-MNs specific genes should not affect the neurodegeneration, and the MNs genes should keep their role as enhancer or suppressor modifiers of the neurodegeneration.

9.5.1. 20 enhancer gene plays its role in MNs

Focusing on the neuronal *enhancer* genes with a general neuroprotective effect, I silenced (2 words omitted) homologs 20 and 31 in the SMA strain sensitive to RNAi only in MNs, to demonstrate whether their neuroprotective effect occurs specifically in MNs (figure 9.7).

The result of the analysis allowed to identify a MNs specific modifier, (2 words omitted) homolog 20. Indeed, when silenced only in MNs, this gene slightly exacerbates the neurodegenerative defect caused by *smn-1* silencing. On the other hand, the other (1 word omitted) homolog, 31, doesn't show the same effect suggesting that its neuroprotective role could be played in another class of neurons.

Both 20 and 31 are poorly studied in *C. elegans* and a very low amount of information are available for both genes. The reason why only one of these candidates play its role in MNs is that only 20 is expressed in MNs and not 31, thus confirming our result.

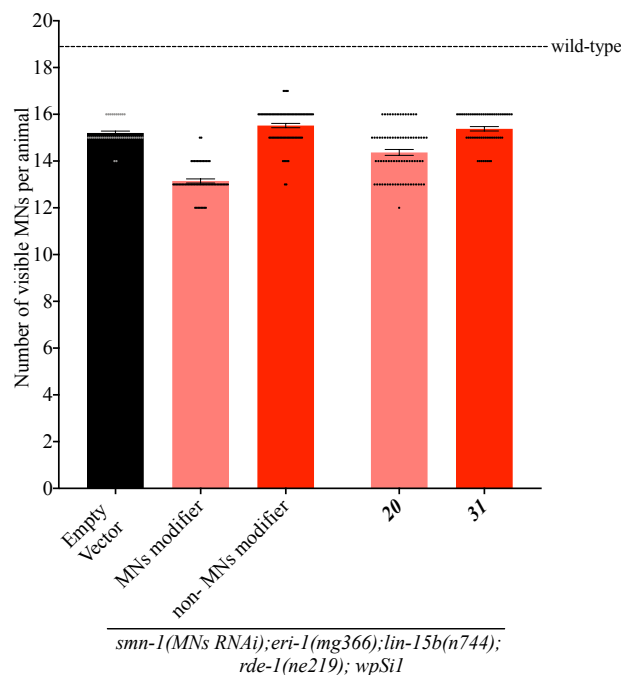


FIGURE 9.7. 20 plays its general neuroprotective specifically in MNs: the graph shows the number of visible MNs of SMA animals sensitive to RNAi in MNs after silencing the neuronal enhancer genes with a general neuroprotective effect. Empty vector (black bar) is the *negative control* of the silencing, and shows the number of visible MNs of animals in which no gene is silenced. The first pink bar (MNs modifier) shows the mean of visible MNs post-silencing of a MNs-specific control gene; the first red bar (non-MNs modifier) shows the mean of visible MNs post-silencing of a non-MNs specific control gene. 20 (pink bar) is a MNs-specific modifiers whose neuroprotective effect occurs specifically in motoneurons since its silencing exacerbate the neurodegeneration caused by *smn-1* silencing ($p < 0.001$ vs Empty vector); 31 (red bar) is a neuronal modifiers playing its neuroprotective role in neurons different from MNs since it doesn't affect the neurodegeneration caused by *smn-1* silencing in this strain ($p > 0.9999$ vs Empty vector). The motoneurons were identified as distinct mCherry-expressing cells and quantified for each animal. The dashed line shows the number of visible MNs in wild-type animals (19). Graphs are scatterplots with bars and whiskers displaying mean and SEM. Each dot represents the number of visible MNs counted per each animal tested. The number of analyzed animal was >50 in each condition. One-way ANOVA with Kruskal-Wallis non-parametric analysis and Dunn's multiple comparison test were performed.

9.5.2. 33 enhancer gene plays its role in MNs

The next step was to determine which of the neuroprotective genes whose enhancer effect is only associated with *smn-1* deficiency plays a specific role in MNs. Thus, I silenced them using the cell-specific strategy (figure 9.8).

The analysis of the neurodegeneration of these enhancer genes allowed the identification of 33 as a new neuroprotective SMA modifier in MNs that deserves further study. *B-RAF/lin-45* also showed a MNs-specific effect. The consistency of this analysis is again supported by the results on *B-RAF/lin-45* and (2 words omitted). With regard to *B-RAF/lin-45*, the cell-specific rescue approach previously described shows that *lin-45* plays a neuroprotective role in MNs, since its over-expression is able to rescue both the neurodegeneration and the locomotion defect caused by *smn-1* deficiency (Hensel *et al.*, 2021); (37 words omitted).

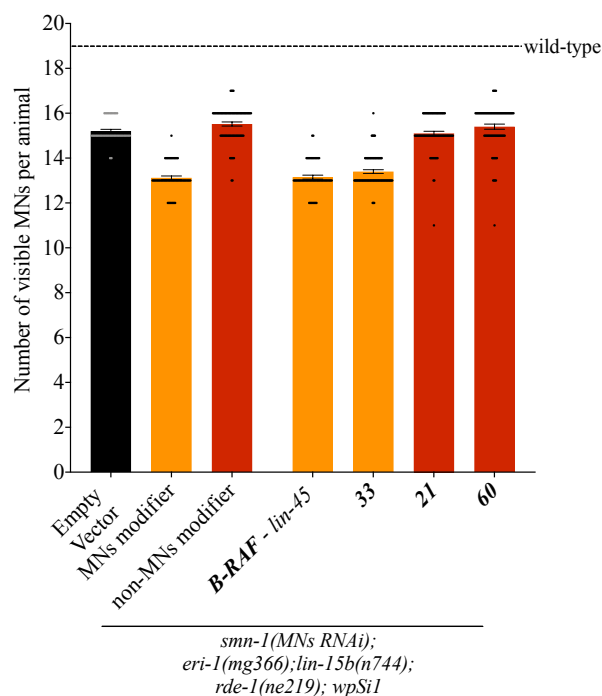


FIGURE 9.8. **33 is a new MNs specific modifier**: the figure represents the pool of 3 experiments performed to silence the 4 enhancer genes with a neuroprotective effect specifically related to *smn-1* deficiency. The graph shows the number of visible MNs of SMA animals sensitive to RNAi only in MNs after the silencing of neuronal-specific enhancer genes. Empty vector (black bar) is the *negative control* of the silencing, and shows the number of visible MNs of animals in which no gene is silenced. The first orange bar (MNs modifier) shows the mean of visible MNs post-silencing of a MNs-specific control gene; the first red bar (non-MNs modifier) shows the mean of visible MNs post-silencing of a non-MNs specific control gene. The orange bars are MNs-specific modifiers whose neuroprotective effect occurs specifically in motoneurons since their silencing exacerbate the neurodegeneration caused by *smn-1* silencing ($p < 0.001$ vs Empty vector); The red bars are neuronal modifiers playing their neuroprotective role in neurons different from MNs since they do not affect the neurodegeneration caused by *smn-1* silencing ($p > 0.05$ vs Empty vector). The motoneurons were identified as distinct mCherry-expressing cells and quantified for each animal. The dashed line shows the number of visible MNs in wild-type animals (19). Graphs are scatterplots with bars and whiskers displaying mean and SEM. Each dot represents the number of visible MNs counted per each animal tested. The number of analyzed animal was > 50 in each condition. The statistical analysis reported was performed for each experiment with the related control performed in parallel. One-way ANOVA with Kruskal-Wallis non-parametric analysis and Dunn's multiple comparison test were performed.

9.5.3. 53 and 51 suppressors play their role in MNs

Finally, I determined which of the suppressor genes acted specifically in motoneurons. Thus, the 9 neuronal suppressors previously selected were silenced in the SMA model following a cell-specific approach (figure 9.9).

The analysis of the neurodegeneration allowed the identification and selection of two new suppressor modifiers with a specific role in MNs: *53* and *51*. *51* role in *C. elegans* MNs is known, (25 words omitted); (14 words omitted).

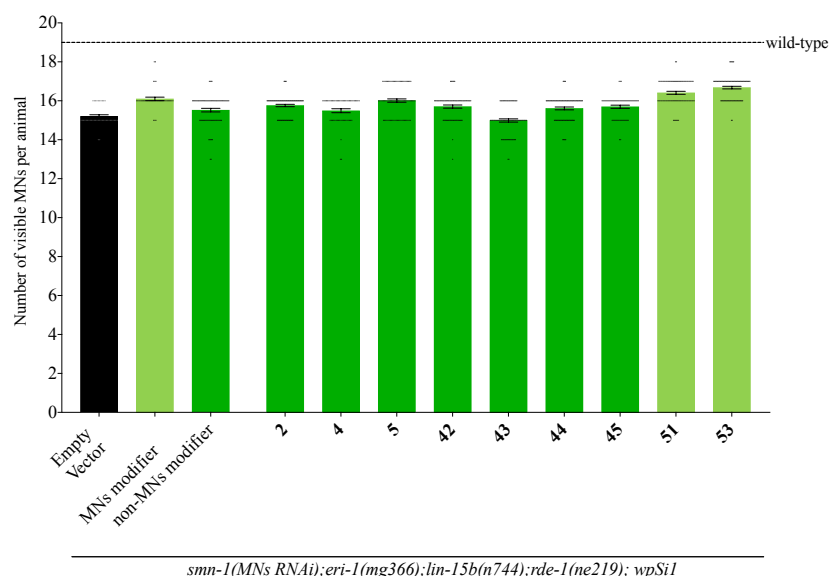


FIGURE 9.9. **53 and 51 are MNs specific suppressors:** the figure represents the pool of 4 experiments performed to silence the 9 neuronal suppressor genes. The graph shows the number of visible MNs of SMA animals sensitive to RNAi only in MNs after the silencing of neuronal-specific suppressors. Empty vector (black bar) is the *negative control* of the silencing and shows the number of visible MNs of animals in which no gene is silenced. The first light green bar (MNs modifier) shows the mean of visible MNs post-silencing of a MNs-specific control gene, the first dark green bar (non-MNs modifier) shows the mean of visible MNs post-silencing of a non-MNs specific control gene. The light green bars are the two MNs-specific modifiers, since their silencing rescues the neurodegeneration caused by *smn-1* silencing ($p < 0.001$ vs Empty vector); the dark green bars are neuronal modifiers playing their suppressor role in neurons different from MNs since they do not affect the neurodegeneration caused by *smn-1* silencing ($p > 0.2$ vs Empty vector). The motoneurons were identified as distinct mCherry-expressing cells and quantified for each animal. The dashed line shows the number of visible MNs in wild-type animals (19). Graphs are scatterplots with bars and whiskers displaying mean and SEM. Each dot represents the number of visible MNs counted per each animal tested. The number of analyzed animal was >50 in each condition. The statistical analysis reported was performed for each experiment with the related control performed in parallel. One-way ANOVA with Kruskal-Wallis non-parametric analysis and Dunn's multiple comparison test was performed.

9.6. *GSK-3/gsk-3* is a general neuroprotective gene with a specific role in MNs

As mentioned in paragraph 9.1, fourteen of the initial candidates homologs to the 37 genes obtained by network biology were essential genes, thus lethal if constitutively silenced. To avoid this pleiotropic effect and to test them anyway, all the essential genes were silenced only in MNs using the cell-specific approach, which should allow to avoid any lethal effect.

First, I silenced the 14 essential genes in the SMA model to identify the genes affecting neurodegeneration when *smn-1* is down-regulated (figure 9.10). Surprisingly, one of this genes, (2 words omitted), also causes lethality when silenced only in MNs. Then, following this approach, a new *enhancer* gene was identified, *GSK-3/gsk-3*, whose role in SMA was partially known since GSK3-inhibitors enhance SMN levels (Chen *et al.*, 2012) and in SMA mice p-GSK3 levels are reduced (Butchbach *et al.*, 2016).

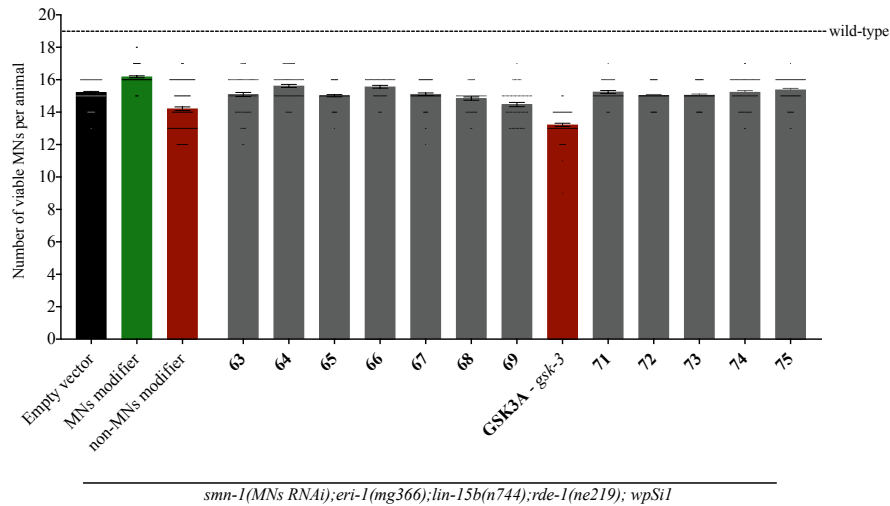


FIGURE 9.10. ***GSK3/gsk-3* is a MNs specific enhancer:** the figure represents the pool of 4 experiments performed to silence the essential genes. The graph shows the number of visible MNs of SMA animals sensitive to RNAi only in MNs after silencing the essential genes. Empty vector (black bar) is the *negative control* of the silencing and shows the number of visible MNs of animals in which no gene is silenced. The green bar (MNs modifier) shows the mean of visible MNs post-silencing of a MNs-specific control gene; the first red bar (non-MNs modifier) shows the mean of visible MNs post-silencing of a non-MNs specific control gene. The grey bars are genes that if silenced do not affect the neurodegeneration caused by *smn-1* silencing ($p > 0.1$ vs Empty vector); the red bar, *GSK3/gsk-3*, is an enhancer gene playing its role in MNs. The motoneurons were identified as distinct mCherry-expressing cells and quantified for each animal. The dashed line shows the number of visible MNs in wild-type animals (19). Graphs are scatterplots with bars and whiskers displaying mean and SEM. Each dot represents the number of visible MNs counted per each animal tested. The number of analyzed animal was >50 in each condition. The statistical analysis reported was performed for each experiment with the related control performed in parallel. One-way ANOVA with Kruskal-Wallis non-parametric analysis and Dunn's multiple comparison test was performed.

Then, to demonstrate if the *GSK-3/gsk-3* neuroprotective effect was general or specifically related to *smn-1* deficiency, I silenced this gene in the wild-type background (figure 9.11). The result of the analysis showed that *GSK-3/gsk-3* neuroprotective effect occurred also when *smn-1* is not mutated, suggesting a general but MNs-specific neuroprotective role of this gene.

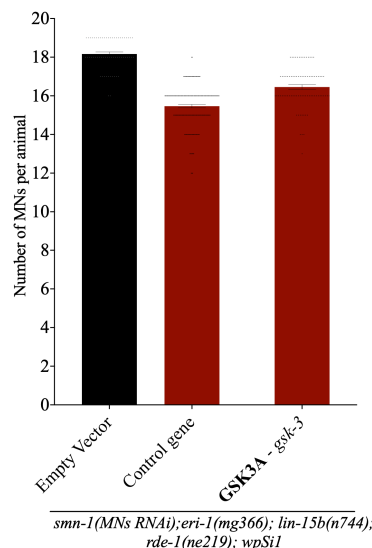


FIGURE 9.11. ***GSK3/gsk-3* is a MNs specific enhancer gene with general neuroprotective effect:** the graph shows the number of visible MNs of wild-type animals sensitive to RNAi only in MNs after the silencing of the essential genes *GSK3/gsk-3*. Empty vector (black bar) is the *negative control* of the silencing and shows the number of visible MNs of animals in which no gene is silenced. The red bar (control gene) shows the mean of visible MNs post-silencing of a MNs-specific control gene. *GSK3/gsk-3* silencing affect neurodegeneration also in this genetic background ($p < 0.01$), suggesting that *GSK3/gsk-3* is generally involved in neuron survival. The motoneurons were identified as distinct mCherry-expressing cells and quantified for each animal. Graphs are scatterplots with bars and whiskers displaying mean and SEM. Each dot represents the number of visible MNs counted per each animal tested. The number of analyzed animal was >50 in each condition. One-way ANOVA with Kruskal-Wallis non-parametric analysis and Dunn's multiple comparison test was performed.

Using different strategies of constitutive and cell-specific RNAi *by feeding*, I investigated the entire network of dysregulated candidates previously identified by Hensel *et al.*, 2021. These approaches allowed the identification of 36 modifiers of neurodegeneration, of which: 1 is an essential and general neuroprotective gene that plays its role specifically in MNs, *GSK-3/gsk-3*; 22 are genes that, when constitutively silenced, enhance the neurodegeneration caused by *smn-1* knock-down; 13 are genes that, when silenced, rescue the neurodegeneration caused by *smn-1* deficiency. Focusing on the 35 non-essential modifiers, 4 of the 13 suppressors play their role beyond nervous system, while the remaining 9 play their role in neurons. Two of these, *51* and *53*, are MNs specific modifiers; on the other hand of the 22 enhancer modifiers, 13 genes are neuroprotective also in *smn-1* wild-type genetic background and one of them, *20* is a MNs specific neuroprotective gene; the remaining 9 were neuroprotective only when *smn-1* is deficient; of these, 5 play their effect beyond nervous system, 4 are neuronal modifiers and *B-RAF/lin-45* and *33* play their neuroprotective effect specifically in MNs (figure 9.12).

Therefore, since *B-RAF/lin-45* neuroprotective effect in SMA was already characterized, we selected the remaining MNs specific candidate genes as the 5 *top target gene* to be further characterized (11 words omitted).

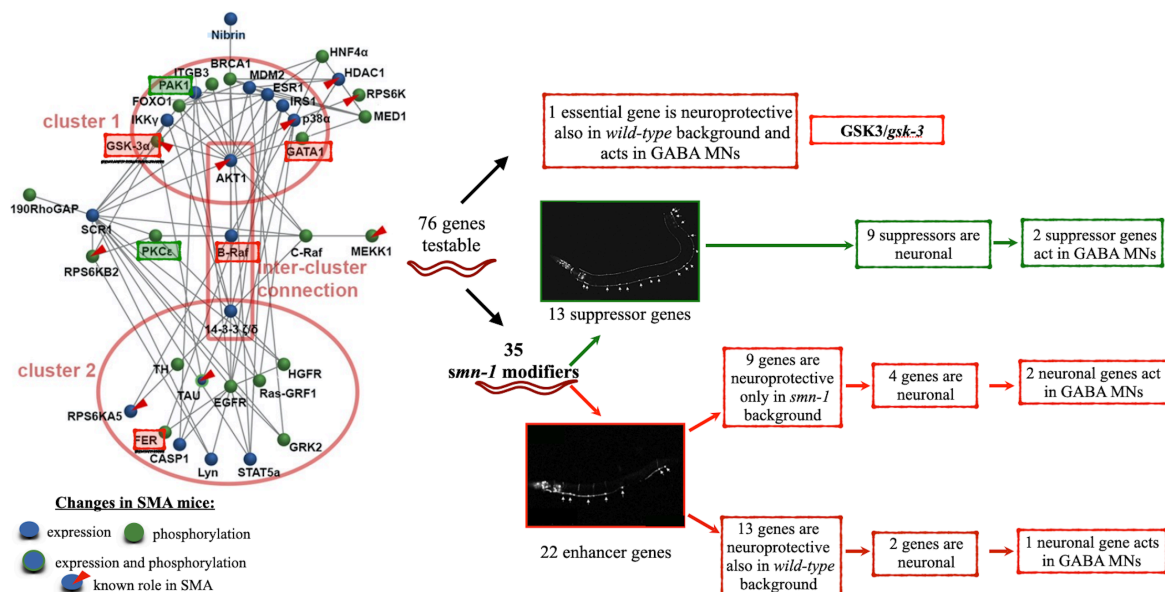


FIGURE 9.12. **Schematic representation of the results of the entire RNAi by feeding screening:** the picture briefly summarizes the results of the screenings performed to select the top candidate genes. Starting from the network identified by Hensel *et al.*, 2021, and using bioinformatics tools, I identified 76 *C. elegans* homologs to the ones belonging to the network, and testable *via RNAi by feeding*. Of the 76 candidate genes, 14 were silenced in MNs and only one of them was selected as enhancer of the neurodegeneration also in wild-type background, *GSK3/gsk-3* (top red box). The remaining 62 genes were constitutively silenced and 35 modifiers were identified, with 13 being suppressors of the neurodegeneration (green boxes), 22 enhancers of the neurodegeneration (red boxes). Of the 22 enhancers, 13 are neuroprotective also in wild-type genetic background (dark red boxes), and 9 are neuroprotective only in *smn-1*(MNs RNAi) genetic background. Following the tissue/cell-specific approach I discovered that 9 suppressors were neuronal modifiers and two of them play their role specifically in MNs (*51* and *53*); 6 enhancers were neuronal-specific and three of them played their role specifically in MNs. Among them 20 is a general neuroprotective gene, and other 2, *B-RAF/lin-45* and *33* are neuroprotective genes only in *smn-1*(MNs RNAi) genetic background.

CHAPTER 10

Confirming the RNAi *by feeding* screening results on *top target* genes

The RNAi *by feeding* strategy allowed the investigation of the whole network designed by Hensel *et al.* 2021. This genetic screening let me identify 35 *modifiers* involved in SMA that will be further characterized to elucidate their role in the disease. Before starting to elucidate their role in SMA and investigate the pathways in which they are involved, it was crucial to confirm the data obtained from the screening with a different approach, in order to demonstrate the consistency of the results. Thus, since the disease primarily affects MNs, I first confirmed the results of the screening using genetic mutants of the two most promising new modifiers, *51* and *53*. Both are neuronal suppressors playing their role specifically in MNs.

10.1. *51*

(105 words omitted)

As mentioned above (see Chapter 9), when silenced, *51* partially rescues the neurodegeneration caused by *smn-1* deficiency, acting as a *suppressor*. Particularly, its suppressor function is played in MNs. In order to verify the result of the RNAi *by feeding* screening and confirm the *51* suppressor effect in the SMA model, a mutant in this candidate gene, *cy*, was tested.

cy is a loss of function allele characterized by a missense mutation altering a conserved residue in the kinase domain of the protein, resulting in a severe kinase activity impairment by altering the structure of the ATP-binding pocket (4 words omitted). To confirm the *51* suppressor nature, I evaluated the effect of this loss of function allele in the *smn-1* (*MNs RNAi*) genetic background on two different phenotypes: the loss of visible MNs and the defect in locomotion.

10.1.1. *51* loss of function mutation doesn't cause neurodegeneration

Before evaluating *51* loss of function in *smn-1*(*MNs RNAi*) genetic background, I wondered whether *cy* causes any neurodegeneration. Thus, I quantified the number of visible MNs in *cy* animals and compared it with wild-type control animals (figure 10.1).

The analysis of the number of visible MNs demonstrated that loss of *51* function does not cause neurodegeneration, since the mean number of visible MNs counted, 18.72, was not different from that of wild-type controls (18.72). This result suggests that *51* is not essential for neuronal survival.

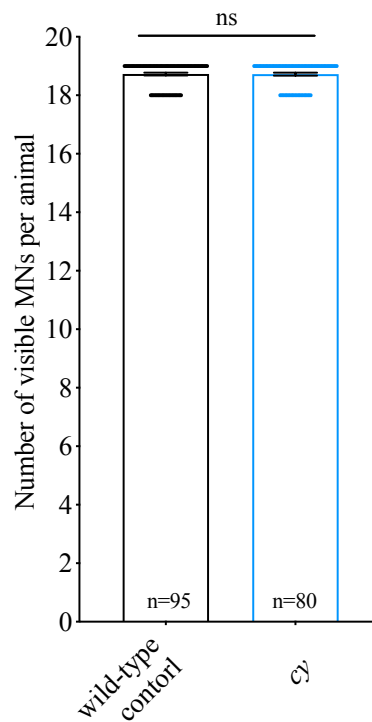


FIGURE 10.1. *cy* does not cause the loss of visible MNs: the graph shows the number of visible MNs animals of wild-type control (black bar) animals and *cy* (light blue bar) strain. The number of visible MNs is not affected in *cy* animal, suggesting that the loss of function of this gene doesn't affect neuron survival. The motoneurons were identified as distinct mCherry-expressing cells and quantified for each animal. Graphs are scatterplots with bars and whiskers displaying mean and SEM. Each dot represents the number of visible MNs counted per each animal tested. n is number of analyzed animal. *t-test* non-parametric analysis and Mann-Whitney test compare rank were performed (ns p=0.6689).

10.1.2. *51* loss of function mutation partially rescues the neurodegenerative phenotype caused by *smn-1* deficiency

Since *51* doesn't affect worm neurodegeneration itself, it is possible to analyze *51* loss of function effects on *smn-1* neurodegenerative phenotype to confirm both the RNAi *by feeding* screening results and its involvement in SMA pathogenesis. Thus, through a genetic cross, I obtained a new strain bearing *cy* in *smn-1(RNAi)* background. Then I analyzed the neurodegeneration of these animals (figure 10.2).

The analysis of the neurodegeneration confirmed the *51* suppressor effect previously assessed through the RNAi *by feeding* screening. When added to *smn-1(MNs RNAi)* genetic background, *51* loss of function mutation partially rescues the neurodegenerative defect caused by *smn-1* silencing. The mean of visible MNs, indeed, increased from 11.66 of the SMA model, to 15.23 of *smn-1(MNs RNAi); cy* strain. This result supports *51* involvement in SMA pathways.

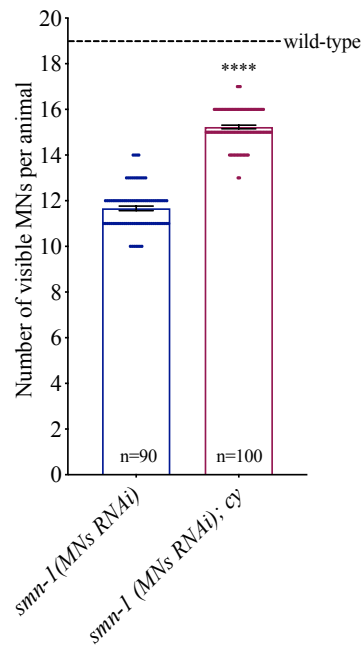


FIGURE 10.2. ***cy2* partially rescues the neurodegeneration cause by *smn-1* deficiency:** the graph shows the number of visible MNs animals of *smn-1(MNs RNAi)* animals (blue bar) and in *smn-1(MNs RNAi)* strain with *cy* (purple bar). The number of visible MNs increase in *smn-1(MNs RNAi);cy* animals compared to the SMA model, confirming *5l* suppressor effect. The motoneurons were identified as distinct mCherry-expressing cells and quantified for each animal. The dashed line shows the number of visible MNs in wild-type animals (19). Graphs are scatterplots with bars and whiskers displaying mean and SEM. Each dot represents the number of visible MNs counted per each animal tested. n is number of analyzed animal. t-test non-parametric analysis and Mann-Whitney test compare rank were performed ($p < 0.0001$).

10.1.3. *5l* loss of function mutation ameliorates the defect in locomotion caused by *smn-1* deficiency

Data obtained from analyzing the neurodegeneration caused by *smn-1(RNAi)* demonstrated that *5l* is involved in the SMA pathway and confirmed this gene effect as a *suppressor modifier*. To further elucidate *5l* effect in the *smn-1(MNs RNAi)* genetic background, I investigated whether *5l* loss of function was able to suppress also the backward locomotion defect caused by *smn-1* deficiency (see Chapter 3). Thus, I performed the backward locomotion behavior assay (figure 10.3).

The backward locomotion assay confirms *5l* suppressor effect also at functional level. *5l* loss of function, indeed, is also able to partially rescue the locomotion defect caused by *smn-1* deficiency ($p = 0.0017$ vs *smn-1(MNs RNAi)*). However, *cy* effect on backward locomotion wasn't tested, thus further analysis are required.

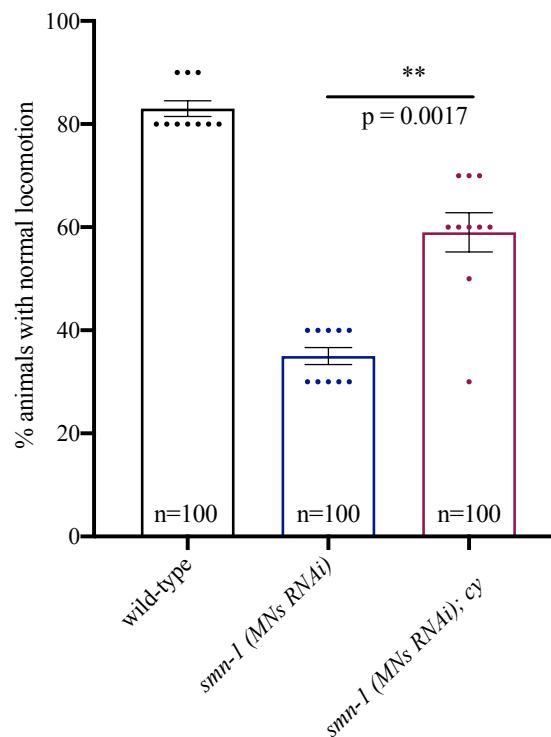


FIGURE 10.3. **51 partially rescues the defective locomotion caused by *smn-1* deficiency:** the graph shows the percentage of animals tested for backward locomotion and able to correctly perform the movement. The animal tested were wild-type animals (black bar), *smn-1(MNs RNAi)* strain (blue bar) and *smn-1(MNs RNAi)* strain also carrying *cy* (purple bar). *51* loss of function also suppresses the defect in locomotion caused by *smn-1* deficiency ($p = 0.0017$ versus *smn-1(MNs RNAi)*). Graphs are scatterplots with bars and whiskers displaying mean and SEM. Each dot represents the percentage of animals among 10 tested with normal locomotion. n is the total number of worms analyzed. One-way ANOVA with Kruskal-Wallis non-parametric analysis and Dunn's multiple comparison test was performed on the raw data as described in materials and methods.

10.2. 53

(109 words omitted)

As mentioned above (see Chapter 9), when silenced, *53* partially rescues the neurodegeneration caused by *smn-1* deficiency, acting as a *suppressor* modifier, playing its suppressor functions specifically in MNs. In order to verify the results of the RNAi by feeding screening and confirm the *53* suppressor effect in the SMA model, I tested a loss of function mutant of this candidate gene, *nj*. *nj* is a putative *53* null allele characterized by a missense mutation of a conserved amino acid in the catalytic loop in the kinase domain. As observed for *cy*, also *nj* impairs the protein kinase activity (8 words omitted) To confirm the *53* suppressor nature, I evaluated the effect of this loss of function allele in the *smn-1 (MNs RNAi)* genetic background on two different phenotypes: the loss of visible MNs and the defect in locomotion.

10.2.1. 53 loss of function mutation doesn't cause neurodegeneration

Before evaluating *53* loss of function in *smn-1(MNs RNAi)* genetic background, I wondered whether *nj* causes itself any neurodegeneration. Thus, I counted the number of visible MNs in *nj* mutants and compared it with wild-type control animals (figure 10.4).

The analysis of the number of visible MNs demonstrated that loss of *53* function does not cause neurodegeneration, since the mean number of visible MNs counted, 18.69, was not statistically different from that of wild-type controls (18.59). This result suggests the *53* is not essential for neuronal survival.

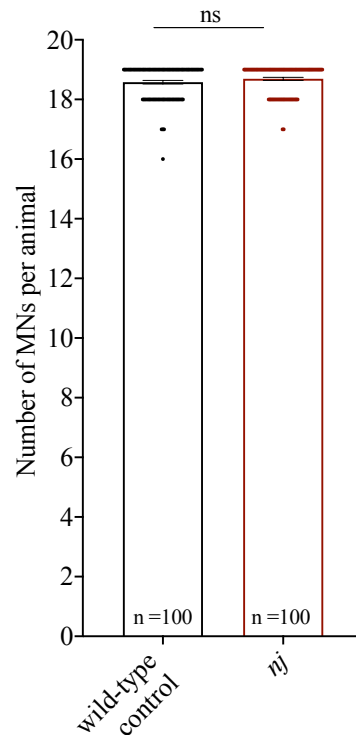


FIGURE 10.4. *nj* does not cause any loss of visible MNs: the graph shows the number of visible MNs of wild-type control (black bar) animals and *nj* (dark burgundy bar) strain. The number of visible MNs is not affected in *nj* animal, suggesting that the loss of function of this gene doesn't affect neuron survival. Graphs are scatterplots with bars and whiskers displaying mean and SEM. The motoneurons were identified as distinct mCherry-expressing cells and quantified for each animal. Each dot represents the number of visible MNs counted per each animal tested. n is number of analyzed animal. *t-test* non-parametric analysis and Mann-Whitney test compare rank was performed ($p = 0.2144$).

10.2.2. 53 loss of function mutation partially rescues the neurodegenerative phenotype caused by *smn-1* deficiency

Similarly to 51, 53 doesn't affect worm neurodegeneration as well. Therefore, I analyzed 53 loss of function effect on *smn-1* neurodegenerative phenotype both to confirm the RNAi by feeding screening result and its involvement in SMA pathogenesis. Thus, through genetic cross, I obtained a new strain with *nj* in *smn-1(RNAi)* background. Then, the neurodegeneration was analyzed (figure 10.5).

The analysis of the neurodegeneration confirmed the 53 suppressor effect previously observed through the RNAi by feeding approach. In *smn-1(MNs RNAi)* genetic background, 53 loss of function mutation partially rescues the neurodegenerative defect caused by *smn-1* deficiency. The mean of visible MNs, indeed, increases from 11.87 of the SMA model, to 14.95 of *smn-1(MNs RNAi); nj* strain. This result supports 53 involvement in SMA pathway.

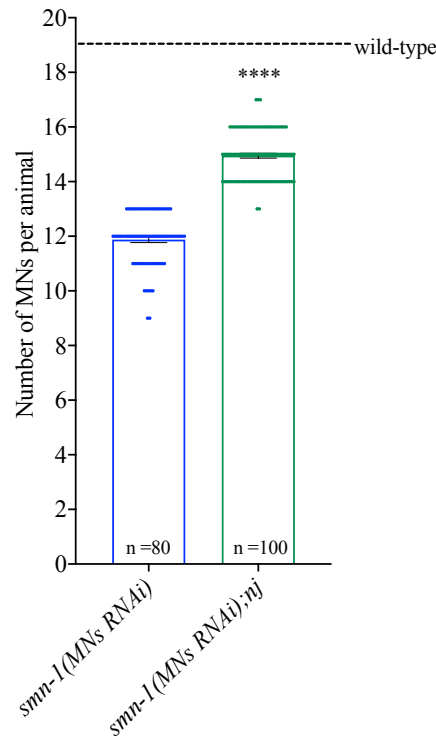


FIGURE 10.5. ***nj* partially rescues the neurodegeneration caused by *smn-1* deficiency:** the graph shows the number of visible MNs of *smn-1(MNs RNAi)* animals (blue bar) and in *smn-1(MNs RNAi)* strain with *nj* (green bar). The number of visible MNs increases in *smn-1(MNs RNAi);nj* animals, compared to the SMA model, confirming *53* suppressor effect. The motoneurons were identified as distinct mCherry-expressing cells and quantified for each animal. The dashed line shows the number of visible MNs in wild-type animals (19). Graphs are scatterplots with bars and whiskers displaying mean and SEM. Each dot represents the number of visible MNs counted per each animal tested. n is number of analyzed animal. t-test non-parametric analysis and Mann-Whitney test compare rank were performed ($p < 0.0001$).

10.2.3. *53* loss of function mutation ameliorates the defect in locomotion caused by *smn-1* deficiency

The data obtained from analyzing the neurodegeneration caused by *smn-1(RNAi)* demonstrated that *53* is involved in the SMA pathway and confirmed this gene effect as a *suppressor* modifier. To further elucidate the *53* effect in the *smn-1(MNs RNAi)* genetic background, I also investigated whether *53* loss of function was able to suppress the backward locomotion defect caused by *smn-1* deficiency (see Chapter 3). Thus, I performed a backward locomotion behavior assay (figure 10.6). The backward locomotion assay confirms *53* suppressor effect also at functional level. *53* loss of function, indeed, is also able to partially rescue the locomotion defect caused by *smn-1* deficiency ($p < 0.0001$ versus *smn-1(MNs RNAi)*). However, *nj* effect on backward locomotion wasn't tested, thus further analysis are required.

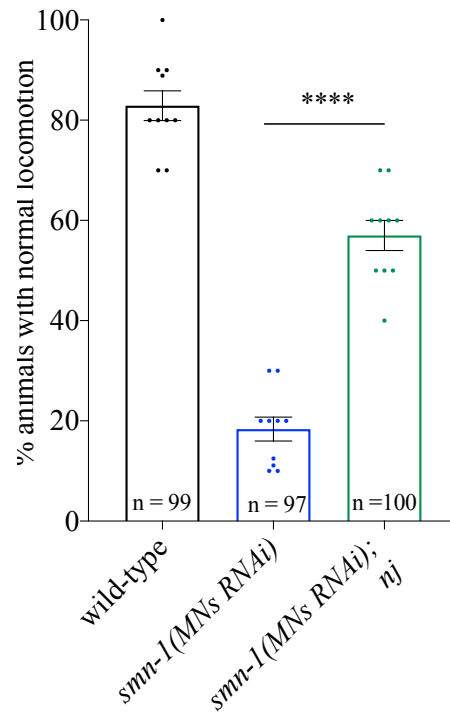


FIGURE 10.6. ***nj* partially rescues the defective locomotion caused by *smn-1* deficiency:** the graph shows the percentage of animals, tested for backward locomotion, able to correctly perform the movement. The animal tested were wild-type animals (black bar), *smn-1(MNs RNAi)* strain (blue bar) and in *smn-1(MNs RNAi)* strain also carrying *nj* (green bar). *53* loss of function also suppresses the defect in locomotion caused by *smn-1* deficiency ($p < 0.00001$). Graphs are scatterplots with bars and whiskers displaying mean and SEM. Each dot represents the percentage of animals among 10 tested with normal locomotion. n is the total number of worms analyzed. One-way ANOVA with Kruskal-Wallis non-parametric analysis and Dunn's multiple comparison test was performed on the raw data.

These genetic approaches confirm the efficiency and validity of the RNAi by feeding screening approach, allowing to select these two genes as top candidates to be better characterized *in vivo* in *C. elegans* and in mammalian SMA models.

CHAPTER 11

Investigating the new SMA *modifier genes* effect in other SMA models

As mentioned in Chapter 3, RNA efficiency could be increased by spreading events mediated by the multi-pass transmembrane protein SID1, which allows dsRNA to spread into neighboring cells (May and Plasterk, 2005). This feature could be critical and limiting when using a cell-specific RNAi approach, such as in the SMA model I used, *smn-1 (MNs RNAi)*, where *smn-1* silencing should be restricted to MNs. In this chapter are showed the results obtained to test whether the spreading events might play a role in the *smn-1(MNs RNAi)* SMA model and a possible strategy to adopt to avoid these undesired events. Moreover, since a slight spreading event occurs in this SMA model, to further confirm the results of the RNAi by feeding screening and, at the same time, collect additional information on the candidate genes found, I tested the candidate hits on two other genetic SMA models: a severe SMA model, *smn-1(ok355)*, and a mild SMA model, *smn-1(cb131)*.

11.1. Spreading events occur in the *gfp(MNs RNAi)* model

Previous results, not showed in this dissertation, demonstrated that the cell-specific RNAi driven by transgenes approach used to silence *smn-1*, is specific for the targeted 19 D-type MNs, since it wasn't observed any spreading effect of the silencing in the nearby cholinergic neurons. However, it was never tested before a possible spreading from MNs to other non-neuronal tissues. Thus, to test whether a spreading of RNAi is in place in the *smn-1(MNs RNAi)* SMA model, I adopted an indirect method. Specifically, I used the same genetic strategy used to silence *smn-1* in MNs (Gallotta *et al.*, 2016; Esposito *et al.*, 2006), to silence GFP in MNs. Subsequently, through a genetic cross, I obtained new strains expressing both GFP in muscle or intestine, the two main *C. elegans* tissues apart from nervous system, and also the transgene causing GFP silencing in MNs. Then I assessed whether a spreading effect occurs by observing if the GFP signal decreased in muscle and intestine.

11.1.1. RNAi spreading occurs from MNs to intestine

The intestine is one of the major organs of *C. elegans*, accounting for one third of the total body mass. It is involved in the digestion and assimilation of food, as well as the synthesis and storage of macromolecules. Due to its large size, the *C. elegans* intestine is also involved in the transport and delivery of various molecules, including dsRNA in RNA interference process. Since its main involvement is in dsRNA transport, I wondered whether the GFP silencing in MNs by RNAi is specific to MNs or also occurs in the intestine. Therefore, I performed a genetic cross to obtain a new strain expressing GFP in the intestine and a transgene that silences GFP in theory only in MNs. Then a phenotypic analysis was performed to assess the GFP expression (figure 11.1).

Phenotypic analysis showed a strong reduction in the intestinal GFP signal when the green fluorescent protein was knocked out in MNs, suggesting a spreading effect from motor neurons to the intestine. Indeed, 90% of the non-silenced animals expressing GFP in the intestine showed a strong and complete signal (see Figure 11.1A, top panel) and only the 10% of them showed a partial GFP signal in the intestine. On the other hand, none of the animals expressing GFP in the gut together with the motor neuron-specific silencing of the same protein showed a complete GFP signal in the gut, something that was not expected (see Figure 11.1A, bottom). Only 20% of them showed a partial GFP signal, but the remaining 80% showed a complete absence of the GFP signal. Further analysis are required to understand and possibly avoid the spreading effect.

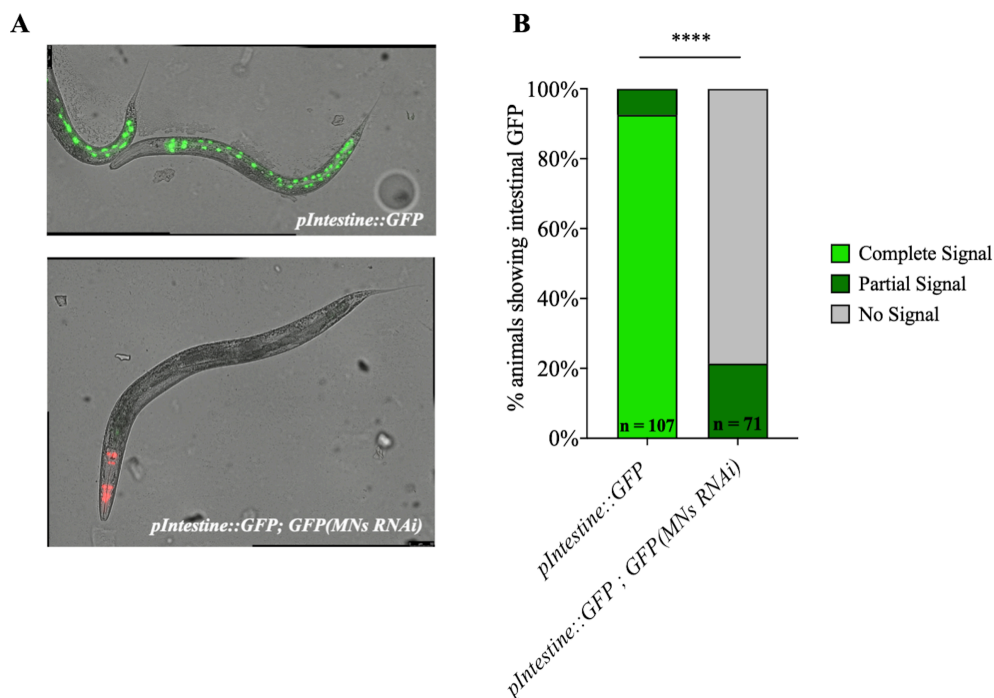


FIGURE 11.1. **Silencing spreading effect from MNs to intestine:** the picture represents the spreading effect tested from MNs to intestine. **A. Picture above:** representative image of *C. elegans* expressing GFP under the *pelt-2* promoter, which exclusively drives expression in the intestine (*pIntestine-GFP*). **Picture below:** representative image of *C. elegans* expressing GFP in the intestine (*pIntestine-GFP*), and the transgene that mediates GFP silencing in MNs, *GFP(MNs RNAi)*. *GFP(MNs RNAi)* expression marker is *pchs2::dsRed2* expressed in the pharynx. **B.** The graph shows the percentage of animals with intestinal expression of GFP when GFP is not silenced (first bar), or when GFP is silenced in MNs (second bar). Light green represents animals expressing a complete GFP intestine signal; dark green segments represent animals expressing a partial GFP intestine signal; grey segment represents animals non expressing GFP in the intestine. n is the number of animals tested. Compare two proportion was performed as statistical analysis to compare the % of animal showing complete GFP signal, partial GFP signal or no signal between the two strains analyzed. **** ($p < 0.0001$).

11.1.2. RNAi spreading occurs from MNs to muscles and it is hampered by *sid-1* loss of function mutation

Muscles are essential for nematode movement and therefore survival. Since SMA is a neuromuscular disease and both muscles and neurons are affected, it is also important to determine whether the transgene that silences *smn-1* in MNs also causes spreading to muscles. Therefore, I made a genetic cross to generate a new strain expressing GFP throughout the muscle system and the transgene that silences GFP in MNs. Phenotypic analysis was then carried out to assess the distribution of GFP in muscles (Figure 11.2 A).

The result of the phenotypic analysis showed a slight reduction of the GFP signal in the muscles when GFP is silenced in the MNs, suggesting a small spreading effect from the motor neurons to the muscles. Indeed, in animals without GFP silencing, 90% of the worms showed a strong and complete signal and only the 10% showed a partial GFP signal. On the other hand, in animals with GFP silencing in MNs and expressing GFP in muscles, 40% of the worms tested show a strong and complete signal, 40% of them show a partial signal and the remaining 20% do not show any GFP signal in muscles.

As mentioned above, the RNAi silencing spreading effect is mediated by multi-pass transmembrane proteins such as *sid-1*. Therefore, a possible adaptive strategy to avoid spreading events could be using loss of function mutations of this gene. To test this hypothesis, I used the loss-of-function *sid-1* allele, *sid-1(qt9)*, to determine whether the slight spreading effect observed from MNs to muscles could be prevented (figure 11.2 B). *sid-1(qt9)* is a non-sense mutation encoding for a truncated nonfunctional protein (Winston *et al.*, 2002).

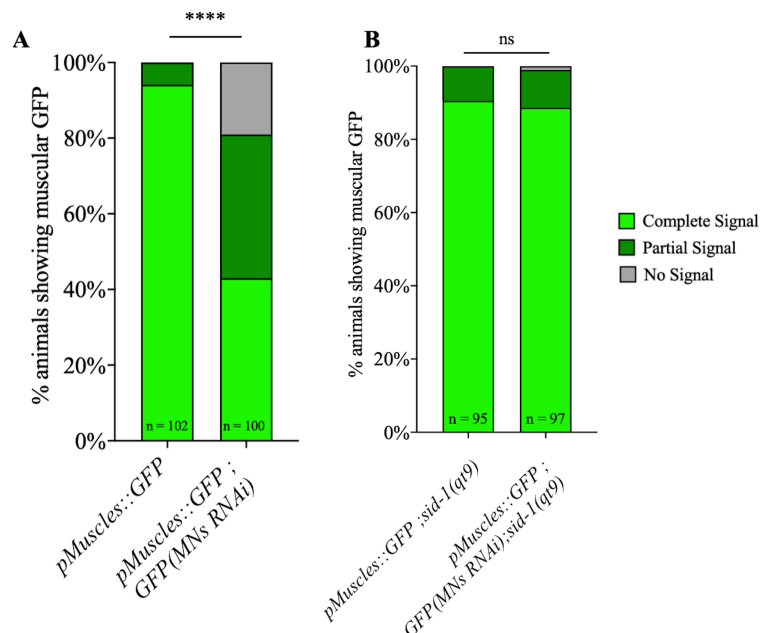


FIGURE 11.2. Silencing spreading effect from MNs to muscle is hampered by *sid-1* loss of function mutation: the graphs represent the spreading effect tested from MNs to muscles. **A.** The graph shows the percentage of animals showing muscular GFP when GFP is not silenced (first bar), or when GFP is silenced in MNs (second bar). Light green segments represent animals expressing a complete GFP muscular signal, dark green segments represent animals expressing a partial GFP muscular signal, grey segment represents animals non expressing GFP in the muscles. **B.** The graph shows the percentage of animals showing muscular GFP when GFP is not silenced (first bar), or when GFP is silenced in MNs (second bar). Light green segments represent animals expressing a complete GFP muscular signal, dark green segments represent animals expressing a partial GFP muscular signal, grey segment represents animals non expressing GFP in the muscles. n is the number of animals tested. Compare two proportion was performed as statistical analysis to compare the % of animal showing complete GFP signal, partial GFP signal or no signal between the two strains analyzed. **** (p<0.0001); ns (p>0.9999).

The analysis confirmed the role of *sid-1* in controlling spreading events, since *sid-1(qt9)* prevents spreading from MNs to muscles. Indeed, 80% of animals belonging to both strains analyzed showed a strong and complete GFP signal in muscles and only the 10% showed a partial GFP signal. These results allowed to confirm the existence of spreading of dsRNA from MNs to muscles and the identification of a possible strategy that could be adopted to prevent it and thus to further implement the *smn-1(MNs RNAi)* SMA model.

11.2. Analysis of the candidate genes using others *C. elegans* SMA models

Although indirectly, previous experiments have shown a possible spreading of the silencing construct from MNs to the intestine and muscles in the SMA model *smn-1(MNs RNAi)*. However, since the *smn-1(MNs RNAi)* SMA model was used to investigate the network obtained by Hensel *et al.* and promising and interesting results were obtained, in order to further confirm them and obtain additional information on all the identified modifiers, I tested the 36 *modifiers* of neurodegeneration in the mild SMA model, *smn-1(cb131)* and the four non-neuronal suppressors in the severe SMA model, *smn-1(ok355)*.

11.2.1. *smn-1(ok355)* lifespan isn't rescued by the non-neuronal suppressor genes

First, as SMA has recently been recognized as a systemic disease, I investigated whether the four non-neuronal candidate genes capable of suppress the neurodegeneration could extend the strongly reduced lifespan of the severe SMA model insensitive to RNAi in neurons, *smn-1(ok355)* (see Chapter 3 paragraph 3.3.2). An RNAi *by feeding* approach was thus adopted, and the candidate genes were knocked-down. The tested genes were: 38, 39, 49, and 54 (figure 11.3).

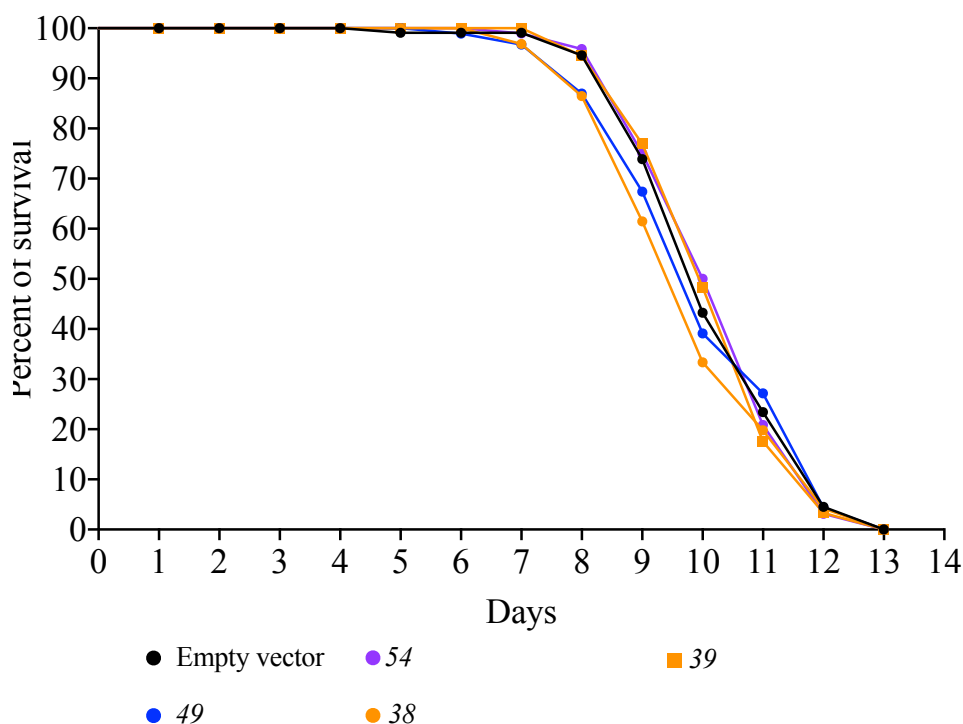


FIGURE 11.3. *smn-1(ok355)* lifespan is not affected by 38, 39, 49, and 54 silencing: the graph shows the percentage of animal survival in *smn-1(ok355)* SMA model after silencing of 49 (blue curve), 39 and 38 (orange curves), or 54 (purple curve). Empty vector (black curve) is the *negative control* of the silencing and shows the percentage of animals surviving when no gene is silenced. None of the genes tested changes *smn-1(ok355)* lifespan ($p=0.5366$). Long-term Mantel Cox test was performed as statistical analysis.

The silencing of the non-neuronal suppressors has no effect on the tested phenotype, and the *smn-1(ok355)* lifespan is not extended after their knock down. In all conditions tested, indeed, the

smn-1(ok355) animals lifespan is 13 days long. These results suggest that these genes could not be involved in this process, but further studies are required.

11.2.2. *smn-1(cb131)* thrashing defect was modified by the non-neuronal suppressor genes

Then, I wondered whether the 36 *positive hits* I identified are also able to modulate the locomotor defect of the *C. elegans* SMA mild model, *smn-1(cb131)* (see Chapter 3 paragraph 3.3.2). All the candidates were firstly silenced through RNAi *by feeding* in the SMA model insensitive to RNAi in neurons. The result of the analysis is reported in figure 11.4.

The thrashing assay performed in the SMA model *smn-1(cb131)* insensitive to RNAi in neurons, showed that among the 36 gene tested, only 5 affect the thrashing defect caused by *smn-1* impairment. Particularly, 4 *suppressors* and 1 *enhancer* gene were identified. Interestingly, all the genes able to affect the thrashing defect caused by *smn-1* deficiency, were also able to modify the neurodegenerative defect caused by *smn-1* silencing and were previously selected as non-neuronal *modifiers*. Furthermore, their role of suppressors or enhancers is conserved in both SMA models tested.

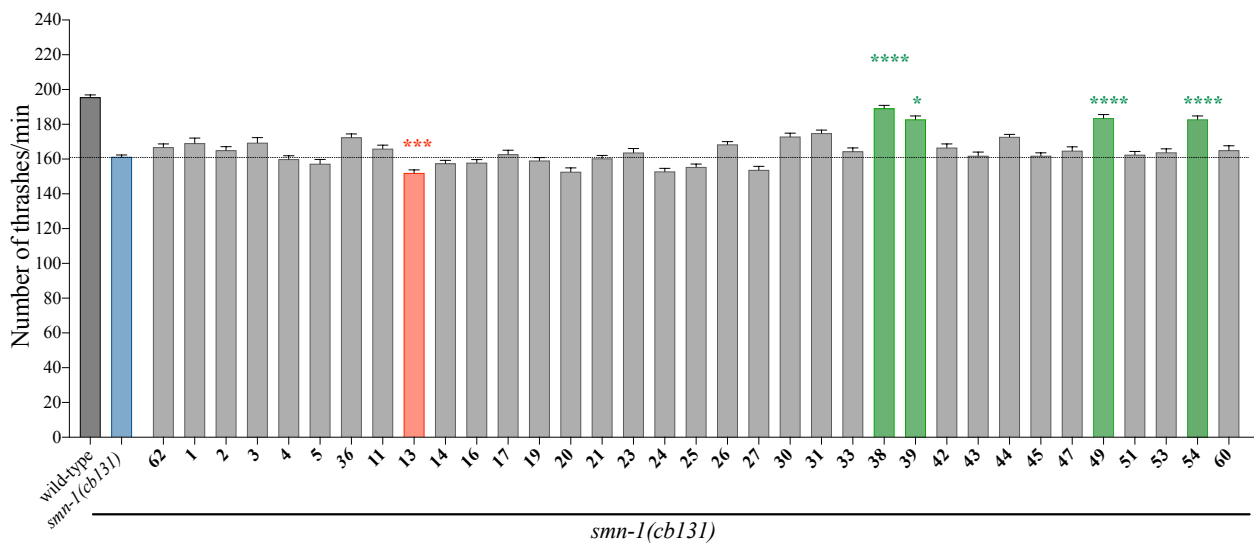


FIGURE 11.4. **SMA modifiers affecting *smn-1(cb131)* thrashing defect:** the figure represents the pool of 8 experiments performed to silence 35 candidate genes and *lin-45*. The graph shows the number of thrashes that a single worm performs in one minute. The black bar shows wild-type animals in which no genes are silenced, the blue bar shows the number of thrashes/min in the SMA model when no gene is silenced. The red bar represents gene that when silenced worsen the defect in thrashing locomotion. The green bars represent genes that when silenced rescue the defect. The grey bars are gene that when silenced do not affect the locomotion defect caused by *smn-1* loss of function mutation. The thin dashed line represents the mean of thrashes/min of *smn-1(cb131)* animals. The number of analyzed animal was >90 for each condition. The statistical analysis reported was performed for each experiment with the related control performed in parallel. One-way ANOVA with Kruskal-Wallis non-parametric analysis and Dunn's multiple comparison analysis. **** (p<0.0001 versus Empty vector); *** (p=0.0004 versus Empty vector); * (p<0.01 versus Empty vector).

CHAPTER 12

Confirming the screening results in mammal SMA models

Because of the high homology to human genes and the many advantages as a model organism, *C. elegans* has proven to be a useful tool for modeling human diseases, including spinal muscular atrophy. However, the use of *C. elegans* alone, while useful for identifying new target genes and molecular mechanisms underlying a disease, is not sufficient. To perform a more complete analysis and make a significant contribution to the study of a disease, it is essential to demonstrate that what I discovered in the nematode is conserved in mammals and therefore applicable to humans. In this regard, the two suppressor target genes identified by the RNAi by feeding approaches and confirmed by genetic mutants, *51* and *53*, were also studied in mammal SMA models: mice spinal cord and mice and human derived cell lines.

12.1. (3 words omitted)

(376 words omitted and figures 12.1 and 12.2)

The RNAi by feeding strategies adopted in *C. elegans* showed that *51* knock-down, suppresses the neurodegeneration caused by *smn-1* silencing. *51* role on the neurodegeneration is played in MNs. These results were confirmed using a *51* genetic loss of function mutant, *cy*. This mutation in *smn-1*(MNs RNAi) genetic background is able to rescue the loss of visible MNs and the locomotion defect caused by *smn-1* deficiency.

12.1.1. Phospho-array results are not confirmed in Taiwanese mice

Phospho-array analysis of Taiwanese SMA mice was performed on the spinal cords of mice at different stages by Hensel *et al.*. Two pre-symptomatic stages P1 (postnatal day 1) and P3 (postnatal day 3) were chosen, and P5 (postnatal day 5), when symptoms occur, to confirm that the changes observed persist at the onset of the disease as well. Analysis showed that (1 word omitted) protein levels were reduced in the spinal cord of SMA mice compared to heterozygous animals, and an up-regulation of the phosphorylated form of (4 words omitted) was also observed. Thus, I wondered whether the phospho-array results could be validated by analyzing (1 word omitted) and (1 words omitted) protein levels in spinal cord of heterozygous and SMA mice. I started by analyzing animals at pre-symptomatic stage P3 (postnatal day 3), but first, as control, I confirmed a decrease in SMN protein levels (figure 12.3).

Since SMN protein levels are reduced in SMA mice, I evaluated the expression levels of the (1 word omitted) proteins and the expression levels of the proteins (3 words omitted and figure 12.4). (92 words omitted and figure 12.4)

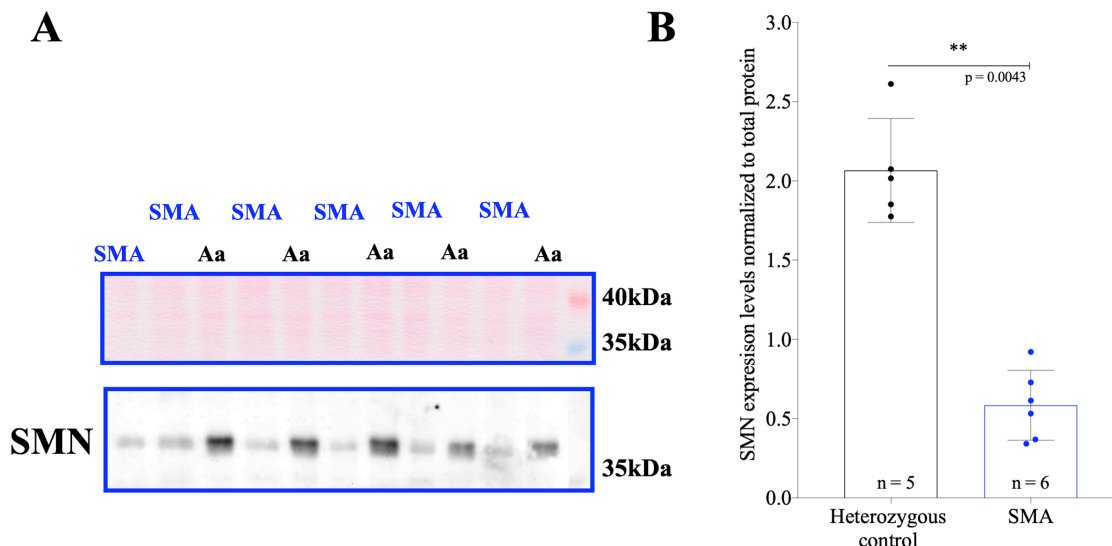


FIGURE 12.3. **SMN protein levels are reduced in P3 spinal cord of Taiwanese SMA:** the figure shows SMN quantification in spinal cord of P3 Taiwanese mice heterozygous (black) and homozygous for *Smn1* mutations (blue). Heterozygous and homozygous SMA mice were siblings, so they shared the same genetic background. **A.** SMN Western blot. The image above shows the Ponceau staining, which represents total protein obtained from each sample tested. The black text marks samples derived from Heterozygous animals (Aa) and the blue text marks samples derived from homozygous SMA mice (SMA). On the marker lane are labelled the bands around the SMN size (40kDa and 35kDa). The image below represents SMN detection at 38kDa. **B.** The graph shows SMN expression levels normalized to total protein. The black bar is the amount of SMN in heterozygous animals, the blue bar is the amount of SMN in homozygous SMA mice. SMA expression levels are reduced in the SMA model compared to control animals ($p = 0.0043$). Graphs are scatterplots with bars and whiskers displaying mean and SD. Each dot is a different biological replicate. n is the number of biological replicates tested. t-test non-parametric analysis and Mann-Whitney test compare rank was performed.

Although the phospho-array results weren't confirmed for this new candidate gene, further studies are needed. Interestingly, the Western blot results are indeed consistent with what has been observed in *C. elegans*, where loss of (1 word omitted) homolog, *51*, has been shown to suppress neurodegeneration caused by *smn-1* deficiency, suggesting an up-regulation of this gene in the SMA model.

12.1.2. (1 word omitted) proteins expression levels are not affected in the SMA murine neuron-like cell line NSC34

The phospho-array analysis performed on Taiwanese SMA mice showed that (1 word omitted) protein levels were reduced in SMA mice. Thus, since I needed to select a cell line to further investigate *in vitro* (1 word omitted) role in SMA, I first tried to confirm the phospho-array result by evaluating (1 word omitted) protein levels in a murine cellular SMA model, NSC34.

The NSC34 cell line is a hybrid mouse motoneuron (MNs) cell line (Cashman *et al.*, 1992), that can be differentiated in neuron-like cells. In 2017, G. Viero's group adopted this cell line to develop a SMA model, silencing at different levels SMN protein. Particularly, following the CRISPR-Cas9 technology, they performed a stable SMN knockdown in NSC34 cells isolating cell lines expressing wild-type (as control) (NSC34 wt), or 20% of full-length SMN (NSC34 20%) (Bernabò *et al.*, 2017).

I evaluated (1 word omitted) protein level in NSC34 wt and NSC34 20% cell lines by performing a Western blot in differentiated neuron-like cells (figure 12.5 omitted).

The analysis showed that in the NSC34 SMA model, (1 word omitted) levels are unaffected. Indeed, Western blot quantification of (1 word omitted) protein levels showed a slight but not statistically significant increase in (1 word omitted) expression levels in NSC34 20% compared to NSC34wt, not confirming what observed in the phospho-array. Further studies are required to confirm this result, and the possibility that the differentiation process wasn't fully efficient must also be considered. Differentiation into neuron-like cells is canonically carried out using retinoid acid; nonetheless, to differentiate NSC34 wt and NSC34 20% cell lines I adopted a method consisting in cells starvation. This method could be less effective than the retinoid acid and could thus affect proteins expression levels including (1 word omitted).

12.1.3. Risdiplam treatment does not affect (1 word omitted) expression in fibroblast derived from patients

One of the aims of the project was to determine whether the identified candidates are SMN independent. Thus, to further investigate the role of (1 word omitted) in SMA and their interaction with SMN, I wondered if modulating SMN expression could affect (1 word omitted) expression. A good strategy to easily modulate SMN expression could consists in treating human cell lines with Risdiplam (Evrysdi®; Roche, Basel, Switzerland). Risdiplam is one of the three FDA and EMA approved treatments for SMA; it acts as an SMN2 splicing modulator, promoting the inclusion of SMN2 exon 7 and consequently the translation of full-length SMN protein. By analyzing whether the expression of (1 word omitted) is altered upon changes in SMN expression, could be possible to determine its dependence on SMN. If (1 word omitted) expression is affected by SMN levels, it suggests it's an SMN-dependent target. Conversely, if (1 word omitted) expression remains unchanged, it could be considered SMN-independent.

I therefore worked with two different patients derived fibroblast cell lines: fibroblast Healthy ML35 and fibroblast Type 0. Thus, a I treated fibroblasts derived from Type 0 patients with 1µM Risdiplam (Evrysdi®; Roche, Basel, Switzerland) for 24h. Not much is known about these two cell lines including the SMN2 copy number. First, I evaluated SMN transcript and protein levels of fibroblast Healthy ML35, and of treated and untreated fibroblasts Type 0, to confirm the efficacy of the treatment. Thus, I confirmed Risdiplam efficiency in increasing SMN levels, since both transcripts (figure 12.6A) and protein levels (figure 12.6C and 12.6D) are increased after the treatment. Moreover, I also confirmed Risdiplam efficacy in modulating SMN2 splicing, since the expression of SMN transcript lacking of the exon 7 is strongly reduced after treatment compared to the untreated cell line (figure 12.6B).

Since I have demonstrated that the Risdiplam treatment works, because SMN levels are changed after treatment, I evaluated (1 word omitted) transcript and protein expression levels in this setting. First, I evaluated (4 words omitted) transcript levels in fibroblasts Healthy ML35, untreated fibroblasts Type 0 and fibroblasts Type 0 treated with 1µM Risdiplam (Evrysdi®; Roche, Basel, Switzerland) for 24h (figure 12.7 omitted).

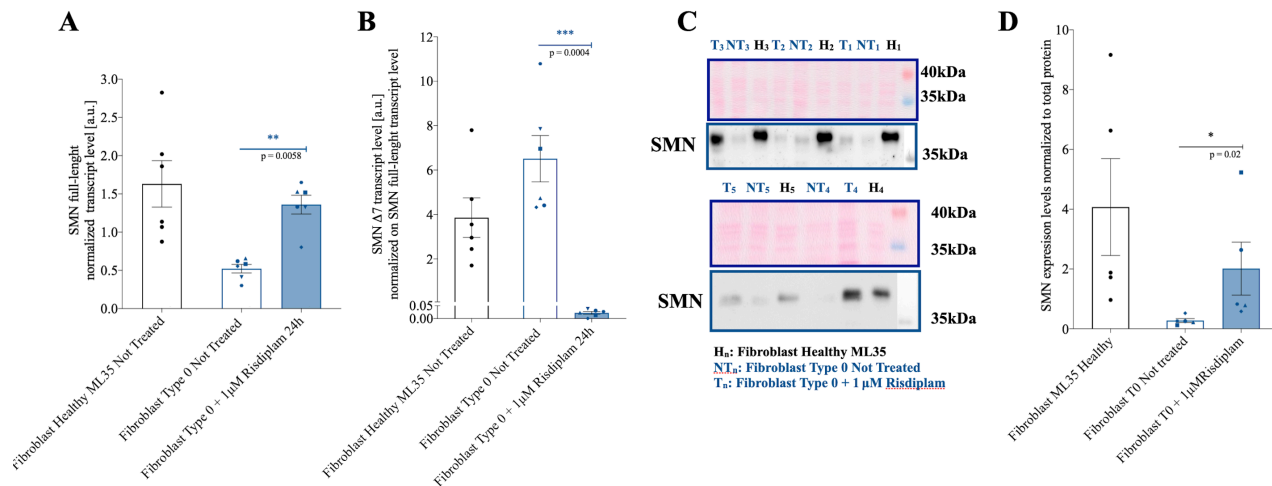


FIGURE 12.6. SMN expression levels in fibroblast cell lines: the figure shows SMN transcript and protein levels in fibroblast Healthy ML35, fibroblast derived from Type 0 patients not treated and treated with 1 μ M Risdiplam for 24h. **A.** The graph shows SMN full-length transcript levels in healthy untreated fibroblasts (black bar), Type 0 untreated Fibroblast (blue bar), Type 0 fibroblasts treated with 1 μ M Risdiplam for 24h (blue filled bar). Risdiplam treatment increases the SMN full-length transcript levels ($p = 0.0058$). Graphs are scatterplots with bars and whiskers displaying mean and SD. Each dot is a different biological replicate, and 6 biological replicates were analyzed. In treated and untreated fibroblast Type 0, dots with the same shape are samples belonging to the same biological replicate and as consequence to the same treatment as well. One-way ANOVA with Kruskal-Wallis non-parametric analysis and Dunn's multiple comparison analysis was performed between treated and untreated type 0 fibroblasts. **B.** The graph shows the amount of SMN transcript lacking exon 7 (SMN $\Delta 7$) in healthy untreated fibroblasts (black bar), Type 0 untreated fibroblasts (blue bar), Type 0 fibroblasts treated with 1 μ M Risdiplam for 24h (blue filled bar). Risdiplam treatment strongly reduced the exon 7 skipping ($p = 0.0004$). Graphs are scatterplots with bars and whiskers displaying mean and SD. Each dot is a different biological replicate, and 6 biological replicates were analyzed. In treated and untreated fibroblast Type 0, dots with the same shape are samples belonging to the same biological replicate and as consequence to the same treatment as well. One-way ANOVA with Kruskal-Wallis non-parametric analysis and Dunn's multiple comparison analysis was performed between treated and untreated type 0 fibroblasts. **C.** The images in the dark blue squares show the Ponceau staining, which represents total protein obtained from each sample tested. All the "H_n" (in black) mark samples derived from fibroblast Healthy ML35, the "NT_n" (in blue) mark samples derived from untreated fibroblasts Type 0, and the "T_n" (in blue) mark samples derived from fibroblasts Type 0 treated with 1 μ M Risdiplam for 24h. On the marker are labelled the bands around the SMN size (40kDa and 35kDa). The images in the blue square represent SMN detection. SMN is 38kDa. **D.** The graph shows SMN expression levels normalized to total protein in healthy untreated fibroblasts (black bar), Type 0 untreated fibroblasts (blue bar), Type 0 fibroblasts treated with 1 μ M Risdiplam for 24h (blue filled bar). Risdiplam treatment increases SMN expression levels ($p = 0.02$). Graphs are scatterplots with bars and whiskers displaying mean and SD. Each dot is a different biological replicate, and 5 biological replicates were analyzed. In treated and untreated fibroblasts Type 0, dots with the same shape are samples belonging to the same biological replicate and as consequence to the same treatment as well. One-way ANOVA with Kruskal-Wallis non-parametric analysis and Dunn's multiple comparison analysis was performed between treated and untreated type 0 fibroblasts.

Analysis of (1 word omitted) transcript levels showed that Risdiplam doesn't affect any of the (1 word omitted) expression levels; indeed, although (1 word omitted) transcript levels seem to be higher in treated cells compared to untreated ones, this difference is not statistically different ($p = 0.08$). Thus, modulation of SMN does not affect the expression of (1 word omitted). Although modulating SMN with Risdiplam has no effect on (1 word omitted) transcript expression levels, this does not mean that it has no effect on (1 word omitted) protein expression. Therefore, I wondered whether treating Type 0 fibroblast with 1 μ M Risdiplam for 24h could affect (1 word omitted) protein expression levels and (2 words omitted) expression levels (figure 12.8 omitted). The analysis showed that in fibroblasts only (1 word omitted) is detectable by Western blot, suggesting that only (1 word omitted) protein is translated in fibroblasts. Furthermore, modulation of SMN doesn't affect (4 words omitted) This suggests a possible independence of (1 word omitted) from SMN in fibroblasts.

Further experiments are needed to investigate in detail the role of (1 word omitted) in SMA pathogenesis, and since the phospho-array results were not confirmed, we also decided to investigate the role of the other suppressor modifier identified by the genetic screening in *C. elegans*, 53.

12.2. (5 words omitted)

(327 words omitted and figure 12.9 and 12.10 omitted).

The RNAi by feeding strategy I adopted in *C. elegans* showed that 53 knock down suppresses the neurodegeneration caused by *smn-1* silencing. 53 role in neurodegeneration is played in MNs. These results were confirmed using a 53 genetic loss of function mutant, *nj*. This mutation in *smn-1(MNs RNAi)* genetic background is able to rescue both the loss of visible MNs caused by *smn-1* deficiency and the defect in locomotion as well.

12.2.1. Risdiplam treatment does not affect both (1 word omitted) expression levels in fibroblast derived from patients

I first investigated whether restoring SMN expression with Risdiplam could modulate (1 word omitted) transcript levels in type 0 fibroblasts. Therefore, I treated fibroblasts derived from type 0 patients with 1µM Risdiplam (Evrysdi®; Roche, Basel, Switzerland) for 24 h and evaluated (1 word omitted) transcript levels by qPCR (figure 12.11 omitted).

Although (1 word omitted) transcript level seems higher in treated fibroblast compared to the untreated ones, the statistical analysis showed that (1 word omitted) transcript level weren't affected by Risdiplam treatment. This result could suggest a possible independence of (1 word omitted) from SMN, but further studies are required.

Discussion

One of the most common genetic causes of infant mortality is Spinal Muscular Atrophy (SMA). SMA is a severe, disabling, inherited neurological disorder, characterized by motoneurons degeneration and progressive muscle weakness and atrophy (Nishio *et al.*, 2023). It is caused by mutations in SMN1 gene resulting in a deficiency of its product, the Survival Motor Neurons protein (SMN), involved in RNA metabolism, regulation of cytoskeletal dynamics, cellular trafficking, autophagy processes, and protein homeostasis (Chaytow *et al.*, 2018). Although motoneurons are primarily affected in the disease, the multiple functions of SMN strongly support the growing evidence in patients that SMN affect also other tissues beyond neurons, supporting the multi-systemic nature of SMA (Yeo and Darras, 2020). *SMN1* gene has a paralogue gene, *SMN2*. These genes are quite similar, their most significant difference is a single nucleotide transition in exon 7 of *SMN2*, that generates an intronic splicing silencer. Therefore, *SMN1* produces a full-length SMN protein, whereas the product of *SMN2* is a truncated and unstable protein that is rapidly degraded (Mercuri *et al.*, 2022). *SMN2* is a modifier of SMA, as patients with a high copy number of *SMN2* have a milder phenotype, demonstrating that the disease results from the reduced amount of SMN protein rather than its complete absence (Tisdale and Pellizzoni, 2015).

In this regard, the current FDA and EMA approved treatments for SMA patients focus on modulating *SMN2* expression and are therefore referred to as SMN-dependent treatments. Particularly, in the last years three SMN-dependent treatments have been approved: Nusinersen (Spinraza®; Biogen, Cambridge, MA, USA), Risdiplam (Evrysdi®; Roche, Basel, Switzerland), and Onasemnogene abeparvovec (Zolgensma®; Novartis Gene Therapies, Bannockburn, IL, USA). While Nusinersen and Risdiplam modulate *SMN2* alternative splicing, promoting the conversion of the unstable SMN2 product in a full-length SMN protein, Onasemnogene abeparvovec pursue a gene therapy strategy to reintroduce the *SMN1* gene into patients. While SMN-dependent therapies have shown remarkable efficacy in improving motor function and enhancing quality of life in SMA patients, their financial burden and potential side effects limit their full applicability. In addition, the lack of data on the long-term effects of AVV9-mediated SMN over-expression, the need to address later stages of neurodegeneration beyond SMN restoration and the fact that current SMN-dependent treatments restore SMN levels mainly in the central nervous system (CNS), highlight the urgency of developing novel therapeutic approaches that can enhance existing treatments. In this context, the new generation of therapeutic strategies (Bowerman *et al.*, 2017) aims to develop SMN-independent therapies that can complement existing ones, overcoming the SMN-dependent treatments limitations. In this regard, it is crucial to identify new neuroprotective compound, and pursue approaches capable to identify additional systems and pathways affected by SMA, in order to select new molecular modifiers that can be targeted to complement the currently approved treatment. *In vivo* and *in vitro* models are definitively useful tools for both aims. In this regard, a useful *in vivo* model for SMA is *Caenorhabditis elegans*. Its powerful genetics, with an easily accessible nervous system, and less ethical constraints compared to mammalian models, make this

nematode ideal for studying neurodegeneration and understanding the molecular and cellular mechanisms underlying several neurodegenerative human diseases, including SMA. In 2016, a new *C. elegans* SMA model was generated (Gallotta *et al.*, 2016), based on the cell-specific knock-down of *smn-1* by RNA interference, specifically in GABAergic motoneurons. The transgenic animals show a defective backward movement, a phenotype dependent on GABAergic motoneurons function, and the loss of motoneurons through a degenerative process. The aims of my thesis work have been to identify and characterize new target genes involved in SMA that could be adopted to develop new complementary SMN-independent therapeutic strategies.

Novel compounds protect against neurodegeneration caused by *smn-1* deficiency in *C. elegans*

One of the strategies adopted to develop treatment for Spinal Muscular Atrophy consists in the identification of small molecules and natural compounds that, when added to diet or integrated with existing treatments, can protect from the neurodegeneration or restore neuronal functions. To this purpose, *C. elegans* has proven to be a valid tool to perform drugs screening and identify new neuroprotective molecules such as 4-aminopyridine (4-AP) (Sleigh *et al.*, 2011) or natural compounds as green kiwi fruit (Mazzarella *et al.*, 2019). Thus, I adopted a *C. elegans* SMA model characterized by *smn-1* silencing in MNs, *smn-1(MNs RNAi)* (Gallotta *et al.*, 2016) to evaluate the effect of *Vigna unguiculata* (Cowpea Seed) natural extract on the neurodegenerative phenotype and to confirm previous results on 4 compounds that have been identified as neuroprotective by a high-content drug screening.

Vigna unguiculata (Cowpea Seed), is a leguminous plant belonging to the *Vigna* species cultivated for human consumption (Tripodi *et al.*, 2020). Known for its rich nutritional profile, Cowpea seeds are a wealth of health-promoting compounds. Cowpea low fat and high protein content, together with its wealth of dietary fiber, phenolic compounds, essential minerals and bioactive peptides with antioxidant activity, underly the potential health benefits of this plant (Tripodi *et al.*, 2020). Studies have consistently linked regular consumption of cowpea with a reduced risk of several health problems, including gastrointestinal disorders, cardiovascular disease, hypercholesterolaemia, obesity, diabetes and several types of cancer. Moreover, a recent study performed also in *C. elegans*, demonstrated the *Vigna unguiculata* protective effect also against neurodegeneration, since the seed extract is able to partially rescue the dopaminergic neuron degeneration in a *C. elegans* model of Parkinson disease (PD) (Tripodi *et al.*, 2020). Since it is known the *Vigna unguiculata* role in neuroprotection, the oxidative stress role in SMA neuronal damage, the neuroprotective role of other antioxidant natural extracts in SMA, such as the green kiwi fruit, and how *C. elegans* can significantly contribute in testing and identifying neuroprotective compounds, I wondered whether the antioxidant *Vigna unguiculata* natural extracts were able to rescue also the neurodegeneration of the *smn-1(MNs RNAi)* *C. elegans* SMA model. Thus, I treated animals with two different concentrations of *Vigna unguiculata* aqueous extract this model, demonstrating a dose-dependent neuroprotective effect of Cowpea seed on the SMA model. This result enriches the set of natural compounds that can be adopted as *functional food* to delay the neurodegenerative events associated

with SMA and others. Indeed, *Vigna unguiculata* has also been shown to be neuroprotective in a *C. elegans* PD model (Tripodi *et al.*, 2020), suggesting that cowpea seed may act on general processes involved in neuron survival that are independent from SMN. To confirm this hypothesis *smn-1* expression levels could be tested after the treatment to evaluate whether they are affected by cowpea seed extracts, and other neurodegenerative models can be tested as well. Furthermore, cowpea seed role in wild-type animals should be analyzed as well.

However, the molecular basis of the observed neuroprotective effect have still to be elucidated, as well as the molecules of the extract involved in the neuroprotection. Unexpectedly, indeed, aqueous extract of cowpea may be poor in phenolic compounds, the main actors of antioxidant activity, suggesting that the antioxidant action could be played by other molecules (Tripodi *et al.*, 2020). On the other hand, *Vigna unguiculata* extract contains starch, amino acids and various proteins (Tripodi *et al.*, 2020) which could act synergistically in neuroprotection. This suggests that the *Vigna unguiculata* neuroprotective effect occurs through different molecular events and is mediated by several molecules. Moreover, this result further confirms the efficiency of *C. elegans* as a model organism for identifying neuroprotective compounds and supports the idea that incorporating *Vigna unguiculata* into the diet could protect neurons from degeneration.

As mentioned above, *C. elegans* has been shown to be a perfect model organism for high throughput drug screening to identify a large number of potential new targets for further investigation in a short period of time. In this context, the *C. elegans* SMA model, *smn-1* (*MNs RNAi*), has been successfully used for an unbiased high-content drug screening in collaboration with Prof. D. Medina, Head of the High Content Screening Facility at TIGEM (Pozzuoli, Italy). This strategy allowed testing of an entire drug library of 1280 FDA-approved molecules and the identification of 4 neuroprotective compounds.

To confirm the results obtained after the automated approach and to exclude false positives, these 4 compounds were tested manually. In particular, I reproduced the screening conditions by growing and treating the SMA model in liquid media and then evaluating the number of visible MNs. The results of this analysis partially confirmed the screening results obtained by the automated approach. Indeed, for 3 of the 4 neuroprotective compounds identified, the neuroprotective effect on the *smn-1* (*MNs RNAi*) model was confirmed. The three neuroprotective compounds confirmed were pimozone and two novel compounds under investigation (Compound 3 and Compound 4, see Chapter 6 paragraph 6.2), the names of which can't be revealed as a patent application is pending. Interestingly, the effect of Compound 3 and Compound 4 on neurodegeneration associated with SMA has never been documented before, and their effect in nervous system, possibly related to axon outgrowth, is also poorly understood. Therefore, since they are FDA-approved drugs and already used to treat other pathological conditions, they could be strong candidates for *drug repositioning* strategies, a new promising therapeutic approach for SMA treatment (Menduti *et al.*, 2020).

These results demonstrate the efficacy of *C. elegans* as a tool to perform automated drug screening capable of identifying new neuroprotective compounds for further characterization. This is supported by the already known role of pimozone demonstrated in another *C. elegans* SMA model.

In particular, Doyle *et al.* characterized the *smn-1* allele *gk1189116* generated by the Million Mutation Project. *smn-1(gk1189116)* is a missense mutation that encodes a defective protein. Although these animals show no developmental defects and reach adulthood, they exhibit several SMA-related phenotypes, including reduced lifespan, neurodegeneration, muscle damage and motility defects (Doyle *et al.*, 2020). Doyle *et al.* have shown that pimozide is able to rescue the defect in locomotion and muscle function, possibly by stabilizing the neuromuscular junction (Doyle *et al.*, 2020). Further investigations are ongoing, focusing on the elucidation of the mechanism of action of these new compounds in neuroprotection, defining whether their mechanism of action is SMN-independent, and whether their neuroprotective effect is also observed in mammalian model systems to confirm the putative role of candidates as future treatments.

B-Raf contributes to motoneuron degeneration in Spinal Muscular Atrophy

The development of complementary new therapeutic strategies for Spinal Muscular Atrophy may require the application of multiple approaches that allow the identification of new systems and pathways involved in the disease, which may also help to better understand the molecular basis of the disorder. In this context, network biology could be an efficient method to identify new pathways involved in the disease, how they are connected, and to understand the interactions between proteins involved in the identified pathways. In this scenario, the group of Prof. Claus, with whom a fruitful collaboration was born some years ago, designed a network biology approach that allowed the identification of a new SMA modifier that has been characterized, *B-RAF/lin-45*. The results of this collaboration were partially published in 2021 (Hensel *et al.*, 2021) with our contribution. In particular, they performed a phospho-array screening on the spinal cord of Taiwanese SMA mice and identified 38 (phospho)proteins that were differentially expressed or phosphorylated between SMA and control conditions. The dysregulated (phospho)proteins were then subjected to hierarchical clustering and network analysis. The network forms two main clusters connected by B-Raf as the central inter-cluster node (Hensel *et al.*, 2021). B-RAF is a serine-threonine kinase that belongs to the Ras-RAF-MEK-ERK signalling pathway, which is involved in cell growth, differentiation, survival and senescence. To properly function the protein must be activated by binding to Ras. Once active, B-RAF phosphorylates its substrates, thereby activating them. The MAPK-ERK pathway, including B-RAF, plays an important role in the differentiation of the nervous system. In particular, the ERK pathway appears to interact with SMN in neurites outgrowth (Hensel *et al.*, 2013), providing evidence for a potential involvement of the MAPK pathway in SMA. Our collaborators showed that in SMA mice total B-Raf protein levels as well as its activated form were reduced at pre-symptomatic (P1-P3) and onset (P5) stages. This reduction was confirmed in motoneurons in SMA mice and in patient-derived motoneurons, where B-Raf is critical for motoneurons survival in response to neurotrophic factors (Hensel *et al.*, 2021). We have also confirmed the putative neuroprotective role of the *C. elegans* B-RAF homolog, *lin-45*, *in vivo*. Using a rescue approach, we showed that constitutive over-expression of hyperactive *lin-45* partially rescued the neurodegeneration and locomotor defects caused by *smn-1* deficiency. We also

showed that in *C. elegans* the neuroprotective effect of *lin-45* occurs before and after the onset of neurodegeneration.

Then I further characterized the role of *lin-45* *in vivo*. Firstly, I demonstrated that the *B-Raf/lin-45* reduction was conserved in the *C. elegans* severe SMA model, *smn-1(ok355)*, since *lin-45* transcript levels were strongly reduced. This recapitulates what observed in mammals, confirming a possible interaction between *smn-1* and *lin-45* as well as the validity of *C. elegans* as model organism to investigate *in vivo* *B-Raf/lin-45* role in SMA. Then, to avoid the obstacles posed by the pleiotropic phenotypes observed in this severe model (Briese *et al.*, 2009), I used the *smn-1(MNs RNAi)* *C. elegans* SMA model to perform a cell-specific rescue approach specifically over-expressing a wild-type and a hyperactive form of *lin-45* only in motor neurons. Using this approach, I obtained a dose-dependent and cell-autonomous rescue of the neurodegeneration and of defects in locomotion caused by *smn-1* silencing, with both the wild-type and the hyperactive versions of *lin-45*. More importantly, using a genetic and pharmacological approach, I have also shown that the neuroprotective role of *B-RAF/lin-45* is mediated through the MAPK/ERK pathway. In fact blocking the MAPK/ERK pathway prevents the beneficial effect of *lin-45*. This shows that the degeneration of SMN-deficient motoneurons is partly caused by a lack of Raf expression/activation. However, hyperactive *lin-45* mutants caused a more robust rescue, suggesting the involvement of other Raf-related mechanisms beyond reduced expression (Hensel *et al.*, 2021). In this regard, one of the other hubs of the network, 14-3-3 ζ/δ , a key regulator of the RAF-MAPK signalling, was also less abundant in SMA mice (Hensel *et al.*, 2021). 14-3-3 ζ/δ is critical for B-RAF kinase activity and reduced 14-3-3 ζ/δ expression may indeed interfere with B-RAF full activation, explaining why B-RAF protein is less active in SMA (Hensel *et al.*, 2021). However in *C. elegans*, silencing of the 14-3-3 ζ/δ homologs, *M117.3*, *ftt-2* and *par-5*, didn't affect the neurodegeneration caused by *smn-1* deficiency (see figures 9.1 and 9.10), suggesting that 14-3-3 ζ/δ role in SMA is not conserved and that also other mechanisms controlling *B-RAF/lin-45* activity may also be involved.

Furthermore, as described in Chapter 9 (paragraph 9.3), *B-RAF/lin-45* silencing in wild-type strains through RNAi *by feeding* doesn't affect the number of visible MNs demonstrating that *B-RAF/lin-45* neuroprotective effect is dependent from *SMN1/smn-1*. Thus, the *B-RAF/lin-45* downregulation observed both at transcriptional (in nematodes) and at protein levels (in mammals) (Hensel *et al.*, 2021), may be directly caused by a molecular function of the SMN protein, possibly related to its role in RNA metabolism.

These results confirm that the neurodegenerative events associated with SMA involving *B-RAF/lin-45* are conserved across species and provide important information on the mechanism of action of its neuroprotective effect (Hensel *et al.*, 2021).

Phospho-array and network biology are powerful tools for identifying relevant signaling nodes in SMA. Altered signalling proteins form a network with B-Raf at its center at pre-symptomatic time points in SMA mouse models. The down-regulation of B-Raf is a putative SMA pathomechanism that can be linked to motor neuron degeneration. The regenerative potential of the B-Raf signaling cluster, which includes several regulatory proteins, needs to be investigated in future studies.

RNAi *by feeding* screening set up

To address the devastating effects of SMA, new approaches are needed that will allow us to elucidate the mechanisms underlying the disease, identify therapeutic targets and develop novel therapeutic strategies. Network biology has been shown to be a promising tool to achieve all of these goals and to make a significant contribution to the field of SMA. In support of this, the phospho-array approach developed by our collaborators followed by network biology analysis allowed the identification of 38 dysregulated proteins in Spinal Muscular Atrophy, arranged in two main clusters connected by a main hub represented by *B-RAF/lin-45*, whose role in the disease has been confirmed in mammals and characterized in *C. elegans* (Hensel *et al.*, 2021).

Besides *B-RAF/lin-45*, the other dysregulated proteins belonging to the network have to be confirmed and characterized. Since *C. elegans* has proven to be a perfect complementary model organism for the identification of new SMA modifiers, I chose it to investigate the entire network in order to identify and select the most interesting dysregulated proteins for further studies. As previously described, *C. elegans* is a feasible tool for genetic screenings and for modulating gene expression through RNAi silencing. Thus, I designed an RNAi *by feeding* screening to silence the *C. elegans* genes homologous to the dysregulated candidates of the network. In RNAi *by feeding*, engineered bacteria carrying a plasmid expressing the dsRNA of interest, are used as a food source. As the worms eat and process the bacteria, the dsRNA is released, the RNAi machinery is induced and the gene of interest is silenced (Grishok, 2005). For this purpose, I adopted the SMA model characterized by *smn-1* silencing in MNs, *smn-1(MNsRNAi)*, due to its advantages in the study of neurodegeneration (Gallotta *et al.*, 2016).

Primarily, I worked on setting up the RNAi *by feeding* screening by optimizing the SMA model and speeding up the analysis.

In terms of optimizing the SMA model, while studying the network, I was interested in identifying SMA modifiers able to play their role in the whole body, including neurons. Therefore, I worked on hyper-sensitizing the whole worm body, including the nervous system, to the RNAi silencing. Indeed, despite its usefulness, RNAi has some limitations, mainly related to its efficiency, and the nervous system resistance to silencing (Conte *et al.*, 2017). Modulating the RNAi machinery using genetic mutants of proteins involved in the silencing processes, allows improving the efficacy and efficiency of the silencing. With this purpose, by genetic cross, I obtained a new *smn-1(MNs RNAi)* SMA model hypersensitive to RNAi in the whole worm body, including the nervous system, by introducing in the SMA model genetic background the *eri-1(mg366);lin-15b(n744)* double mutations that increase the RNAi efficiency. Using the same procedure, I also obtained a new SMA model not hypersensitive to RNAi in the whole body which has the same genetic background as the hypersensitive one. Interestingly, possibly due to the new genetic background and unrelated to *eri-1(mg366);lin-15b(n744)* mutations, both new SMA models show a less severe neurodegenerative phenotype compared to the previous SMA model I used, which can be genetically modulated in both directions (improvement and worsening). This allows the identification of genes that can rescue the neurodegenerative phenotype caused by *smn-1* silencing (*suppressor modifiers*) or that can worsen it (*enhancer modifiers*), as demonstrated by the pilot

RNAi *by feeding* experiment performed on control genes. Thus, for the first time, we have obtained new models that allows to identify modifiers that suppress or enhance the neurodegenerative phenotype. This greatly enriches our expertise in using *C. elegans* to study SMA, since we still haven't been able to directly identify *enhancers* of the neurodegeneration. However, the fact that the strains allow to identify *enhancer modifiers* hasn't yet been confirmed using genetic mutants, a more consistent approach than RNAi *by feeding*. Thus, it is planned to test whether a loss of function mutation of the enhancer control gene *WDR79/tcab-1* and of another known SMA enhancer, *PLST3/plst-1*, could exacerbate the neurodegenerative phenotype caused by *smn-1* in the new strains obtained. On the other hand, even though I confirmed the ability of the new SMA model not sensitive to RNAi in the whole body to identify *suppressor modifiers* using genetic mutants, I have yet to confirm the same for the SMA model hypersensitive to RNAi in the whole body something that is also planned.

Time is a critical element in studying a neurodegenerative disease and finding a cure. So when I set up the RNAi *by feeding* screening, I chose the more efficient analysis mode which also optimizes the workload as much as possible. In this regard, I compared the canonical method used in our lab to quantify the neurodegeneration, consisting in anesthetizing worms, preparing slides and then using a compound microscope to count the number of visible MNs per animal, with an alternative method, consisting in analyzing animals directly in the plates avoiding slides preparation and using a stereo microscope; I then evaluated the time taken to complete the analysis. Although I demonstrated that is possible to assess neurodegeneration avoiding slide preparation by analyzing animals directly in the plates thus saving time and opening up to the possibility to observe the same worm over time anesthetizing worms is always required and only 20' are saved overall. This is not advantageous enough to use this method for the whole screening. However, new machine learning technologies and automated counting strategies could contribute to further speed up the screening protocol. In conclusion, the RNAi strategy chosen and set up, as well as the preferred method adopted for analyzing neurodegeneration *in vivo*, together with the fast life cycle of *C. elegans*, allowed me to perform the whole RNAi *by feeding* screening in almost 8 weeks.

An RNAi *by feeding* screening to identify new SMA modifiers

As already mentioned, in addition to *B-Raf/lin-45*, the phospho-array and network biology approach developed by Prof. Claus' group (Hensel *et al.* 2021) allowed the identification of 37 additional dysregulated proteins in SMA, whose role in the disease needs to be confirmed and characterized. In this regard, I set up the RNAi *by feeding* screening and obtained the new SMA model to optimize the screening efficiency.

To validate the 37 proteins *in vivo*, at first, I used Ortholist 2.0 (Kim *et al.*, 2018) to identify the *C. elegans* homologs of the proteins of the network and found that three of them, MED1, IRS1 and IKK γ , have no homologs in *C. elegans* and thus can't be included in the screening. This result were also confirmed using TreeFam - database of animal gene trees (treefam.org) and BLASTP.

IKK γ (NF-kappa-B essential modulator) is a regulatory subunit of the IKK complex involved in the NF-kappa-B signalling pathway, which is mainly involved in controlling immunity and inflammation (uniprot.org). This signalling pathway is partially conserved in *C. elegans*, but the IKK γ nematode counterpart is missing (Kurcz and Ewbank, 2003). However, further studies could be carried out on the pathway. Indeed, since the immune system is known to be involved in SMA (Yeo and Darras, 2020), it might be possible to focus on some IKK γ upstream or downstream targets that have an homolog in nematodes, such as *TRAF6/trf-1* or *I κ B/ikb-1*, and investigate this interaction in *C. elegans* anyway.

MED1 (mediator of RNA polymerase II transcription subunit 1) is a nuclear receptor activator that belongs to the Mediator complex, a multi-protein complex acting as a transcriptional activator (uniprot.org). This structure is evolutionarily conserved among eukaryotes including *C. elegans*. Although none of the bioinformatic tools consulted for homology found a match for MED1, *mdt-1.1* has been predicted to be a putative subunit of the Mediator complex and an homolog of the human MED1 (Grants *et al.*, 2015). Thus, this gene could be also tested in the future.

IRS1 (insulin receptor substrate 1) is a signalling adaptor protein involved in the insulin receptor signalling pathway. It is phosphorylated by the insulin receptor and mediates PI3-K activation triggering the insulin/IGF-1 signaling (uniprot.org). This pathway is fully conserved in *C. elegans* and it is involved in several function including development, aging and behavior (Murphy and Hu, 2005). As in the case of MED1, none of the bioinformatic tools used to search homology found a match for IRS1. However, *ist-1* could be a putative homolog of IRS proteins. Thus these new candidate could also be tested in the next future. Although I didn't include *ist-1* in the RNAi *by feeding* screening, the homologs of other key players in the insulin pathway and *IRS/ist-1* downstream targets have been silenced in the screening: (6 words omitted). These genes silenced in the SMA model do not affect the motoneurons degeneration caused by *smn-1* silencing, suggesting that in MNs the insulin pathway may not to be involved in the neurodegenerative event related to *smn-1* deficiency.

Except for MED1, IRS1 and IKK γ , Ortholist 2.0 search unexpectedly allowed me to identify 83 homologs for the remaining 34 candidates, since some of the candidates had more than one homolog in nematodes. Of course, these 83 *C. elegans* genes are not all orthologs to the 34 genes, but since the screening in *C. elegans* allows to silence a high number of genes in a rapid time, we preferred to start with a larger cohort, adding no bias to the selection.

Of this 83 *C. elegans* homologs, 7 were not testable *via* RNAi *by feeding* because they were not present in the RNAi *by feeding* library adopted for the analysis and available in the lab. However, they can be tested anyway using loss of function genetic mutants. In addition, of the 76 testable remaining genes, 14 were essential genes, thus causing lethality when constitutively silenced, hence not testable for neurodegeneration at young adult stage.

Afterwards, I used the new SMA model hypersensitive to RNAi in the whole body including the nervous system to silence all the testable candidate genes. I completed the screening in almost 8 weeks and identified 35 new *modifiers genes* as putative new SMA *target genes* for further characterization. Of the 35 new *modifier genes*, 22 were *enhancer genes*, i.e. genes that enhanced

the neurodegeneration when silenced and reducing the number of visible MNs compared to the SMA model; 13 were *suppressors*, i.e. genes that suppressed the neurodegenerative defect caused by *smn-1* silencing and increasing the number of visible MNs compared to the SMA model. The remaining 27 candidate genes didn't affect neurodegeneration when silenced, including the previously mentioned (8 words omitted). These results may be attributed to the possibility that these 27 genes and associated pathways are not involved in the neurodegeneration caused by *smn-1* deficiency in MNs, at least in *C.elegans*. Alternatively, it is possible that these genes are unrelated to the survival of motoneurons, and thus their silencing does not impact the specific cell class analyzed. However, given their dysregulation in SMA mice, these genes may still be involved in SMA beyond motoneurons. Consequently the *C. elegans* model employed to perform the screening, designed for investigating motoneurons degeneration may not be ideal for discerning their relevant mechanisms. Thus, to further investigate these genes and test whether they are involved in the SMA pathway and could be used to develop an SMN-independent treatment, a good strategy would be to test them in the severe and in the mild *C. elegans* SMA models. These animals do not show neurodegeneration and the lifespan or the defect in thrashing locomotion could be tested instead. Following this first round of RNAi *by feeding*, I focused on the 35 *modifiers* to characterize them and select the ones with motoneuron specific function for further studies. Since the number of the candidates was still high, I adopted different strategies of constitutive and cell-specific RNAi *by feeding*.

First I focused on the 22 *enhancer genes*. A gene able to worsen the neurodegeneration when silenced is involved in neuron survival. This phenomenon may or may not be related to *SMN/smn-1* neuronal functions and *SMN/smn-1* deficiency. Thus, to distinguish *enhancer genes* involved in neuroprotection even when *SMN/smn-1* expression is not affected, I silenced them in wild-type background. 13 genes out of 22 were able to reduce the number of visible MNs in a wild-type background, causing neurodegeneration. Interestingly, some of these genes are involved in neurodevelopment and axon outgrowth, events that overlap with *SMN/smn-1* functions. Thus a possible synergistic role with the SMA causative gene cannot be excluded. This hypothesis could be demonstrated following a genetic approach. Indeed, genetic crosses can be used to obtain new strains with the same genetic background but expressing the *smn-1(MNs RNAi)* construct, or a loss of function mutation of a *candidate gene*, or both. By scoring the number of visible MNs in these strains and comparing the results, it should be possible to define whether these target genes complement or not with *smn-1*, thus defining their possible interaction.

Of the 22 *enhancer genes* silenced in wild-type background, 9 of them do not affect the number of visible MNs, suggesting that their neuroprotective effect is strongly related to *smn-1* deficiency. Interestingly, with the exception of for *B-RAF/lin-45* whose role in the SMA is discussed in this thesis, none of the other candidate has been reported to have an effect in SMA.

As mentioned before there is evidence of SMA multi-system nature (Yeo and Darras, 2020), thus it may be pivotal to determine whether the 35 *modifiers* effect is specifically played in neurons or whether other tissues are also involved. So, I employed an indirect approach by silencing the 35 *positive hits* in the SMA model insensitive to RNAi in neurons. In this genetic background, a

modifier with a neuron-specific role, will not show any effect on the neurodegenerative phenotype, since neurons are refractory to interference, and neuron-specific genes are not affected by silencing. On the other hand, non-neuronal *modifier* can be efficiently silenced and this will affect the number of visible MNs. This RNAi *by feeding* approach allowed to identify 20 *modifiers* (11 general neuroprotective genes, 5 *smn-1 enhancer* genes and 4 *suppressors*) as non-neuronal ones and the remaining 15 (2 general neuroprotective genes, 4 *smn-1 enhancer* genes and 10 *suppressors*) as neuronal *modifiers*.

Although I haven't focus on the non-neuronal modifiers yet, their investigation could be crucial to discover new druggable targets that ameliorate SMA symptoms beyond the nervous system, complementing the actual treatment of the disease. A non-neuronal *modifier* could both affect MNs degeneration acting from other tissues, or synergistically acting in nervous system and other tissues, like muscles. Unfortunately, the SMA model employed does not allow to distinguish between the two putative mechanisms of action. However, taking advantage of *C. elegans* strains that are hypersensitive to RNAi only in a specific tissue, such as muscles or intestine, it might be possible to determine whether their effect is only non-neuronal or also neuronal.

The remaining 15 *modifiers* are genes that act as *enhancers* or *suppressors* in neurons. These include *B-RAF/lin-45*, which has previously been shown to exert a neuronal-specific neuroprotective role (Hensel *et al.*, 2021), and (2 words omitted) with a known neuronal function (4 words omitted), thus strengthening the efficacy of the approach adopted. Interestingly, this approach identified new unexpected modifiers, (74 words omitted). Although all of the newly identified modifiers are interesting and warrant further investigation, because spinal muscular atrophy is primarily known as a neurodegenerative disease and primarily affects motor neurons, and we are interested in studying neurodegeneration, I chose the neuronal *modifiers* to continue my research. To this end, I silenced the 15 neuronal modifiers specifically in MNs and identified 10 non-MNs *modifier genes* (1 general neuroprotective gene, 2 *smn-1 enhancer* genes and 7 *suppressors*) and 5 MNs *modifiers* (1 general neuroprotective gene, 2 *smn-1 enhancer* genes and 2 *suppressors*).

(40 words omitted). On the other hand, as expected, the 5 MNs *modifiers* include *B-RAF/lin-45*, whose cell-autonomous neuroprotective role has been demonstrated in this work (Hensel *et al.*, 2021). (36 words omitted)

(160 words omitted)

The involvement of (7 words omitted) in SMA could be supported by the fact that they share some common functions with SMN, such as their role in cytoskeleton dynamics, apoptosis and neuronal growth. Furthermore, since all of them are involved in cytoskeletal dynamics and axonal outgrowth, these represent pathways to focus on further.

As mentioned before, 14 of the candidates genes were essential, i.e. lethal if constitutively silenced. Using the same cell-specific RNAi *by feeding* approach, I silenced this extra 14 candidates only in motoneurons, identifying another general neuroprotective gene, *GSK-3/gsk-3*. It encodes for a serine/threonine protein kinase involved in glucose homeostasis, and Wnt signalling. It has a known a role in neurodegeneration and in SMA since is known that GSK3-inhibitors enhance SMN levels

(Chen *et al.*, 2012), suggesting that this gene is SMN dependent thus not attractive to our purpose, since we are more interested in SMN-independent modifiers.

However, the identification of *modifiers* already known to have a role in the SMA, such as *GSK-3/gsk-3*, strengthens both the scientific value of both the phospho-array approach and of the RNAi *by feeding* strategy, as well as the complementary use of different model systems.

Confirming the RNAi *by feeding* screening results on *top target* genes

The role of *GSK-3/gsk-3* role in SMA has been already investigated, thus we decided to focus on the other candidates. (14 words omitted)

(837 words omitted)

Investigating the new SMA *modifier* genes effect in other SMA models

A critical event in RNAi interference is the spread of dsRNA throughout the body to produce a systemic RNAi effect. This mechanism is mediated by specific pathways involving multi-pass transmembrane proteins, such as *sid-1*, which allows dsRNA to spread to neighboring cells (May and Plasterk, 2005). Although spreading is a critical event for increasing RNAi efficiency and could also be exploited for cell-specific RNAi strategies, it could be limiting when using a cell-specific RNAi approach, such as the SMA model *smn-1 (MNs RNAi)*, where *smn-1* should only be silenced in MNs. I therefore further characterized the *smn-1 (MNs RNAi)* SMA model to assess whether a spreading event occurs. Although it was previously demonstrated that the RNAi *as* transgene used to silence *smn-1* is specific to target the 19 D-type MNs, since no spreading event was observed in the cholinergic neurons, a putative spreading event towards other tissues was never tested. Therefore, I used an indirect method, and specifically used the same genetic strategy in place to silence *smn-1* in MNs (Gallotta *et al.*, 2016; Esposito *et al.*, 2007), the RNAi *as* (sense-antisense) approach, but to silence the GFP in MNs; then I evaluated whether this transgene was able to reduce GFP expression due to a spreading event in two of the *C. elegans* tissues, intestine and muscle.

The intestine is one of the major organs of *C. elegans* and is involved in several important functions essential for the survival of this nematode; among them, due to its large size, the *C. elegans* intestine is involved in the transport and delivery of various molecules, including dsRNA in RNA interference. Since the intestine is mainly involved in dsRNA transport, I wanted to investigate whether GFP silencing in MNs by RNAi is specific to MNs or also occurs in the intestine. Therefore, I obtained a new strain that expresses GFP in the intestine and the transgene that silences GFP in MNs. A phenotypic analysis was then carried out to assess GFP expression. Surprisingly the result showed a strong reduction of GFP expression in the intestine, suggesting a spreading event, from neurons to intestine. A similar result was observed in muscle. In fact, by analyzing the strain that expresses GFP throughout the muscle system and the transgene that silences GFP in MNs, I observed a slight spreading effect from MNs to muscles. Interestingly, the spreading event to muscles was prevented using a loss-of-function mutant of the multi-pass transmembrane proteins *sid-1*, *sid-1(qt9)*. This result determines a possible strategy to implement the *smn-1(MNs RNAi)* SMA model and to avoid the spreading effect, but further studies are needed to clarify the spreading

mechanisms. Indeed, although the spreading phenomenon was observed in both intestine and muscle, it appeared to be less penetrant in the latter. This may suggest that the only spreading event of the RNAi construct occurs from MNs to the intestine and not to other tissues, and that what I observed in muscles was only an indirect effect caused by the transport of the silencing construct from the intestine to other tissues. The first hypothesis should be tested by checking in the *sid-1(qt9)* mutant if we can also prevent the spreading effect from MNs to intestine; then, to demonstrate that the main spreading event from MNs to intestine, GFP expression can be assessed in muscles when GFP is silenced in MNs and intestine is resistant to RNAi. However, since *sid-1* is poorly expressed in neurons, we need to investigate how the silencing construct spreads from the MNs. Additionally, also the transgene that silences *smn-1* in MNs needs to be tested. This could be done using the strain carrying the *smn-1* allele, *rt280*. These animals express the ubiquitous *SMN1* protein fused to GFP (O'Hern *et al.*, 2017). Through genetic cross, one could obtain the SMA model *smn-1(MNs RNAi)* also expressing the *smn-1(rt280)* allele in its genetic background and evaluate whether the GFP signal fused to SMN changes in different organs.

(256 words omitted)

Confirming the screening results in mammals SMA models

(1383 words omitted)

Future perspective

The results obtained during my PhD project demonstrate once again the great advantage of using *C. elegans* to study SMA and how the synergistic use of this nematode and *in vitro* mammalian models could significantly contribute to rapidly improving our knowledge of the disease and in finding complementary therapeutic strategies for patients. Particularly, this thesis demonstrated the usefulness of *C. elegans* in rapidly identifying small molecules and natural compounds that can protect MNs from degeneration, and in identifying new target genes involved in SMA that could be modulated to ameliorate the symptoms.

The identification of the neuroprotective effect of *Vigna unguiculata* highlight the putative benefits in using *functional foods* to delay the neurodegenerative events. However, the molecular basis of cowpea neuroprotection remains to be elucidated. In this regard, further analysis of the neuroprotective effect needs to be performed to evaluate whether cowpea extracts improve also worm neuron functions by rescuing the SMA models locomotion defects. Additionally, the molecules and the pathways involved in the neuroprotection related to cowpea treatments need to be identified. In this regard, using *C. elegans* genetics mutants could be useful, as well as performing a transcriptomic or metabolomic analysis in treated and untreated SMA animals to observe how the transcript or metabolic profile might change, thus identifying new target molecules to further analyze. Of course, the *V. unguiculata* neuroprotective effect needs to be confirmed in *in vitro* models and the synergistic effect of this natural extract with the approved drugs can also be tested. For instance, evaluating SMN expression levels in fibroblast cell lines derived from SMA patients untreated, treated with Risdiplam and treated both with Risdiplam and *V. unguiculata* extracts could be an interesting strategy to confirm whether cowpea seed could complement the SMN-dependent treatments. Similar strategies could be used as well, to further analyze the novel compounds identified by the HCS strategy.

The SMN-independent therapies developing pipeline (curesma.org), include the use of approaches allowing to identify new systems and pathways affected in SMA. In this context phospho-arrays and network biology could provide a comprehensive approach. This was reinforced by the identification of the neuroprotective role of *B-RAF/lin-45*, the central *hub* of the network obtained by Hensel *et al.* (Hensel *et al.*, 2021). These results encouraged further investigation, and the RNAi *by feeding* screening was performed. Although 35 new positive hits were identified and we decided to mainly focus on the MNs-specific target genes, the research is open to further analysis. Particularly the neuronal *positive hits* will be tested in other SMA models. In fact, I have obtained by genetic cross the mild SMA *smn-1(cb131)* hyper-sensitized to RNAi in the whole body including neurons. Thrashing behavior assay will be assessed on this strain after silencing to determine which of the neuronal modifiers also affects this phenotype.

In recent years, the interest of the SMA research community has increased in investigating mechanisms beyond MNs, so our studies could be extended beyond the nervous system by focusing on the non-neuronal targets. Cell-specific RNAi by feeding strategies could help in this regard by allowing to the identification of the tissues in which the identified modifiers play their role.

(407 words omitted)

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