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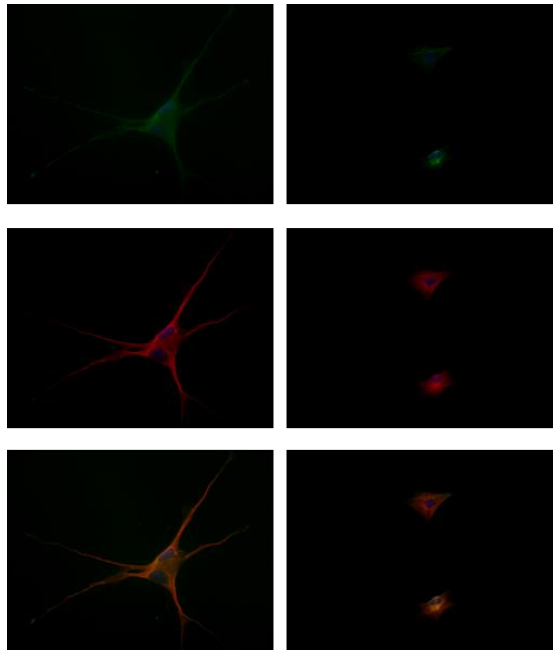
MYC and *MINCR* characterization in ALS:

oncogenes role in neurodegeneration



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A Federica,
che ha reso tutto più bello
(e che mi piace copiare)

TABLE OF CONTENTS

1. ABSTRACT 1
2. ABBREVIATIONS 3
3. INTRODUCTION 4
3.1 Amyotrophic Lateral Sclerosis (ALS) 4
3.1.1 Epidemiology 4
3.1.2 fALS and sALS 5
3.2 Pathogenesis of motor neurons degeneration in ALS 6
3.2.1 Genetics 7
3.2.2 Oxidative stress 9
3.2.3 Protein aggregation 9
3.2.4 Excitotoxicity 10
3.2.5 Mitochondrial impairment 11
3.2.6 Axonal transport dysfunction 11
3.2.7 Contribution of non-neuronal cells in inflammatory process 12
3.2.8 Cell cycle and apoptosis 12
3.3 Transcription alteration in ALS 13
3.3.1 RNA metabolism 13
3.3.2 Noncoding RNAs 15
3.3.3 miRNAs 16
3.3.4 Role of miRNAs in pathogenesis of neurodegeneration 17
3.3.5 Long non-coding RNAs 18
3.3.6 Role of lncRNAs in pathogenesis of neurodegeneration 21
3.3.7 Long noncoding RNAs and ALS 22
3.3.8 Transcriptional factors alterations in ALS 23
3.4 A shared mechanism of RNA metabolism in cancer and neurodegeneration 25
3.4.1 c-MYC 27
3.4.2 MYC and neurodegeneration 30
3.4.3 MYC-induced long non-coding RNA (MINCR) 30
4. AIMS 33
5. MATHERIALS AND METHODS 34
5.1 Patients and control subjects 34
5.2 PBMCs extraction from blood samples 35
5.3 RNA extraction 35
5.4 cDNA synthesis, qPCR and ddPCR 35
5.5 Protein extraction and quantification 36
5.6 Western Blot 37
5.7 Immunoprecipitation IP 38
5.8 Immunofluorescence IF 39
5.9 Chromatin immunoprecipitation ChIP 39
5.10. Cell culture and differentiation 40

5.11. Cell stable transfection and silencing 40
5.12. RNA immunoprecipitation RIP 41
5.13. Library preparation for RNA-seq and bioinformatic data analysis 42
5.14. Pathway analysis and Gene Ontology 42
5.15. RNA Pull down 42
5.16. CRISPR-Cas9 editing 44
5.17. Apoptosis assay 46
6. RESULTS 47
6.1 MYC network alteration in sALS 47
6.1.1 MYC and its molecular partner RNAs are deregulated in PBMCs and spinal cord of sporadic ALS patients 47
6.1.2 The major part of deregulated genes in sALS are MYC targets 48
6.1.3 Protein levels of MYC and its interactors are comparable between sALS patients and healthy controls 49
6.1.4 MYC/Max complex dimerization is impaired in PBMCs of sALS patients 50
6.1.5 MYC subcellular distribution is different in sALS patients 51
6.1.6 MYC binds the TSS region of MINCR less in sALS patients 52
6.2 Characterization of MINCR 53
6.2.1 MINCR and MYC 53
6.2.2 MINCR is a nuclear lncRNA 55
6.2.3 Deep sequencing RNA expression profiles in neuronal cell model carrying over- and down-expression of MINCR 56
6.2.3.1 DEGs subcellular distribution indicates the MINCR action compartment 58
6.2.3.2 GO terms enrichment of deregulated transcripts 59
6.2.3.3 Pathway analysis of deregulated transcripts 61
6.2.3.4 MINCR transcript levels impact on oncogenesis 63
6.2.3.5 MINCR transcript levels impact on neurodegeneration 68
6.3 MINCR and ALS 72
6.3.1 MINCR binds to histone H1 in differentiated SH-SY5Y 72
6.3.2 MINCR knock-out leads to alteration in cytoskeleton and increase in late apoptosis 72
7. DISCUSSION 76
8. REFERENCES 81

1. ABSTRACT

Amyotrophic Lateral Sclerosis (ALS) is a neurodegenerative disease that causes both upper and lower motor neuron degeneration. Despite our knowledge has increased in the last years, ALS still remains a pathology of uncertain etiology characterized by the interplay between many different processes and molecules. One of the emerging involved processes is RNA metabolism, both involving coding and non coding transcripts. Dysregulation in RNA-binding proteins strongly involved in ALS (such as TDP-43 and FUS), in gene expression and transcription have emerged, beside the idea that alterations in long non coding RNAs (lncRNAs) play an important role in ALS pathogenesis. Nevertheless, the exact contribution of RNA metabolism dysregulation to ALS is still unknown. Therefore, we performed a whole transcriptome analysis in Peripheral Blood Mononuclear Cells of sporadic unmutated ALS patients (sALS) compared to healthy controls that revealed two main concepts: a deregulation in genes involved in transcription and a significant alteration of lncRNA expression. In the light of these considerations, we found interesting the deregulation of MYC Binding Protein (MYCBP) and MYC induced long non coding RNA (MINCR) RNA levels in sALS, both related to the transcription factor MYC. Moreover, 75 differentially expressed coding genes (DEGs) out of 87 (86.2%) resulted to be MYC targets. Therefore, we decided to investigate MYC network dividing the project in two main tasks:

1. To identify the causes of MYC transcriptional activity alteration and the consequences on gene expression and RNA metabolism in sALS.

2. To explore lncRNA involvement in the pathogenesis of sALS. For this aim we focused our attention on MINCR, investigating its features, targets and mechanisms of action.

For the first task, we demonstrated that in sALS the MYC/Max heterodimer formation is impaired, probably altering the normal transcriptional activity. As a matter of fact, through a Chromatin Immunoprecipitation assay followed by qPCR, we demonstrated that MYC binds the Transcription Start Site region of MINCR less in sALS patients compared to healthy controls. Since MYC binds MINCR promoter to induce its transcription and since we have demonstrated that there is an impairment in MYC/Max complex, we suggest that this alteration can lead to a minor rate of transcription of MINCR, thus leading to MINCR down-regulation in sALS.

For the second task, we firstly investigated the possible regulatory mechanism exerted by MINCR on MYC network. We demonstrated that neither the overexpression nor the knockdown of MINCR in SH-SY5Y neuroblastoma cell line caused a significative alteration in MYC, MYCBP and Max mRNA expression compared to untreated conditions. Moreover, RNA immunoprecipitation (RIP) of MYC in PBMCs of sALS patients and control subjects followed by RIP-seq and Droplet Digital PCR analysis showed that MINCR does not bind to MYC protein, led us to conclude that the mechanisms of action of MINCR does not involve MYC

directly. RNA-seq analysis of SH-SY5Y cells overexpressing and silenced for MINCR allowed us to identify different deregulated RNAs. From DEGs analysis, KEGG, Wikipathways and GO analysis a clear trend emerged showing a great role for MINCR overexpression in cancer and MINCR down-regulation in neurodegeneration. Finally, we investigated the effect of MINCR in ALS. Firstly, through RNA pull-down assay we demonstrated that MINCR binds directly to Histone H1 protein. Knocking-out MINCR through CRISPR-Cas9 system in differentiated SH-SY5Y cells, we demonstrated that SH-KO cells showed hallmarks of apoptosis, confirmed with Annexin V and 7-AAD staining, which showed that MINCR KO leads to increase in apoptosis, especially in late apoptosis. Moreover, alteration in cytoskeleton were detected in SH-KO after differentiation protocol, showing an impairment in axonal outgrowth.

In conclusion, dysregulation of RNA metabolism appeared to be a central mechanism in sALS. Our data brought the light on transcription activity alteration of MYC, paving a new way for future studies in MYC related pathways and network. Furthermore, we highlighted also lncRNA alteration in sALS, especially demonstrating the mechanisms of action and targets of MINCR that, thus, can be considered a new possible interesting therapeutic target.

2. ABBREVIATIONS

ALS: Amyotrophic Lateral Sclerosis

sALS: sporadic Amyotrophic Lateral Sclerosis

FTD: Fronto-Temporal Dementia

AD: Alzheimer's disease

PD: Parkinson's disease

lncRNA: long non coding RNA

ncRNA: non coding RNA

miRNA: microRNA

PBMCs: Peripheral Blood Mononuclear Cells

MINCR: MYC-induced long non Coding RNA

DEG: differentially expressed genes

TSS: transcription start site

ChIP: chromatin immunoprecipitation

RIP: RNA immunoprecipitation

IP: immunoprecipitation

IF: immunofluorescence

ddPCR: droplet digital PCR

CRISPR-Cas9: Clustered Regularly Interspaced Short Palindromic Repeats-Cas9

3. INTRODUCTION

3.1 Amyotrophic Lateral Sclerosis (ALS)

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease characterized by motor neuron loss in the spinal cord, brainstem and motor cortex [1]. Neuronal degeneration leads to muscular atrophy and spasticity that evolve to paralysis; the disease is fatal within 3-5 years of onset, generally due to respiratory failure [2]. ALS was first described as a clinic-pathological entity by the French neurologist Jean Martin Charcot in 1869. "Amyotrophic" refers to the atrophy of muscle fibers, which are denervated as their corresponding anterior horn cells degenerate, leading to weakness of affected muscles and visible fasciculation. "Lateral sclerosis" refers to hardening of the anterior and lateral cortico-spinal tracts as motor neurons degenerate in these areas and are replaced by gliosis [2]. Indeed, the pathological hallmark of ALS is denervation and atrophy of muscle due to loss of spinal motor neurons. Accumulation of phosphorylated neurofilaments, swelling of the proximal axon and perikarya, Bunina bodies and Lewy body-like inclusions and the deposition of inclusions of ubiquitinated material in these axons are observed [3] [4]. Moreover, astrocytes and microglia activation and proliferation are also common in ALS [4]. Despite the degeneration affecting both upper and lower motor neurons, ALS has traditionally been considered a neuromuscular disease. However, in the past decade clinical, neuropathological and imaging data have showed an extensive involvement of the CNS. Population phenotyping data show that up to 50% of patients with ALS develop cognitive and behavioral impairment and about 13% of patients have concomitant behavioral variant frontotemporal dementia (FTD), a non-Alzheimer's type dementia [5] [6]. So far, there is no primary therapy for this disorder. The only drug approved by the US Food and Drug Administration for the treatment of ALS is the Riluzole, a glutamate antagonist, which mechanism of action is still unclear [7]. Symptomatic measures (as respiratory support and feeding tube) are the main tools for the management of this disorders [2].

3.1.1 Epidemiology

The prevalence of ALS in European populations has been estimated at 2·6–3·0 cases per 100 000 people [4]. In most population-based studies, ALS is found to be more common in men than in women, affecting 1·2–1·5 men for every woman [8]. Lifetime risk is about 1:350 for men and 1:400 for women [9] [10]. In populations of European ancestry, the median age of onset of sporadic ALS is 65 years, whereas the mean age of onset in genetically heterogeneous populations is about 10 years earlier [10]. Moreover, the recent growing recognition of a continuum between ALS and frontotemporal dementia seems to have shifted the types of patients who are included

in epidemiological studies, which could partly explain the observed increase in the incidence of ALS, particularly in people at let stages of life [11].

3.1.2 fALS and sALS

ALS can be caused by inherited genetic mutations (fALS) or occurs sporadically (sALS). The familial cases represent only the 5-10% of patients and they can be inherited either as an autosomal dominant or recessive trait. Adult onset autosomal dominant inheritance is more common than juvenile onset caused by recessive transmission. In one family it has been reported a X-linked dominant inherited ALS [12]. Thanks to increased genome wide sequencing projects, more mutations have been discovered, adding more heterogeneity to the disease mechanisms. Among the genes reported, there is strong evidence supporting a pathogenic role for trans active response DNA-binding protein of 43 kDa (TARDBP) in 3% of cases, fused in sarcoma (FUS) in 5% of cases, Cu / Zn superoxide dismutase 1 (SOD1) present in 20% of cases and a newly identified hexanucleotide repeat expansion in chromosome 9 open reading frame 72 (C9orf72) in 38% of cases [13]. Mutations in these genes cause motor neuron death through different pathways: SOD1 mutations lead to oxidative stress, TARDBP, FUS and C9orf72 induce disturbances in RNA machinery because their principal physiological functions include RNA processing, such as transport, splicing and translation [14]. A single mutation can lead to different clinical presentations and similar ALS phenotypes result from different mutations, implying that ALS is a syndrome of different causes that share similar pathophysiological pathways [15].

A genetic component is also thought to contribute to the pathogenesis of sporadic ALS, which accounts for the majority of ALS cases (90-95%). A meta-analysis of three twin studies gives an estimate of sALS heritability of 0.61 (95% CI 0.38–0.78) [16]. However, the identification of particular gene mutation in sALS cases account for a small number of the total cases, reflecting a complex pattern of inheritance with very low penetrance, a high degree of heterogeneity and the existence of environmental factors predisposing to ALS [17].

Even if ALS is relentlessly progressive, the variability in clinical disease duration is large, with some patients surviving only few months after onset and others surviving for more than two decades. Large differences in survival and age at disease onset exist even between individuals from one family, in whom ALS is caused by exactly the same mutation, suggesting the existence of other factors that modify the phenotype [2]. Although ALS patients show some degree of heterogeneity, in symptoms, age of onset and disease duration, fALS cases are indistinguishable from sALS based on clinical and pathologic criteria. Thus, genetics and family history are the primary factors that discriminate between sALS and fALS [18]. All genes found mutated in fALS cases may also be mutated in sALS and first-degree relatives of patients with sALS have an increased risk of ALS and other neurodegenerative

diseases [17]. As a matter of fact, similar superoxide dismutase 1 (SOD1)-positive and TAR DNA-binding protein 43 (TDP43)-positive inclusions have been found in sALS and fALS [19] [20].

3.2. Pathogenesis of motor neurons degeneration in ALS

As in other neurodegenerative diseases, the exact molecular pathway causing motor neuron degeneration in ALS is still unknown, probably because it is likely to be a complex interplay between multiple pathogenic cellular mechanisms.

Firstly, ALS appears as a multisystemic disease based on the involvement of different cell types and tissue, such as microglia and astrocyte, oligodendrocytes and muscles cells, that are all targeted by the disease processes and actively participate to the final disease outcome [2]. Furthermore, different genes have been identified so far that are associated to ALS pathogenesis and that now cover a large fraction of the familial cases of the disease [15]. Depending on their known biological role or on newly acquired mutated functions, they are connected to different physiological processes, implying that diverse pathogenic mechanisms, not necessarily related, might be involved in ALS. Over the last two decades, cellular biological studies have led to the development of multiple interconnected pathogenic themes in ALS. Excitotoxicity is glutamate-mediated neurotoxicity that has been considered as a possible pathogenic mechanism of ALS even before the identification of SOD1 as a causative gene. During this process excessive synaptic glutamate triggers calcium overload in postsynaptic neurons, likely inducing mitochondrial dysfunction. Other involved pathways are based on the fact that MutSOD1 may interact or associate to the mitochondria, the ER and the glial Nox2/Rac1 complex, therefore resulting in mitochondrial dysfunction, ER stress and ROS overproduction, respectively. Motor neuron injury derived from mitochondrial impairment, dysregulation of calcium, protein aggregates formation handling with proteasome impairment and ER stress, leading to activation of the apoptotic cascade. Moreover, disrupted axonal transport and loss of metabolic support from myelin sheaths seem to contribute to motor axon vulnerability. Finally, non-cell autonomous processes, such as prion-like propagation of MutSOD1 and neuroinflammation, might also damage motor neurons (Figure 1).

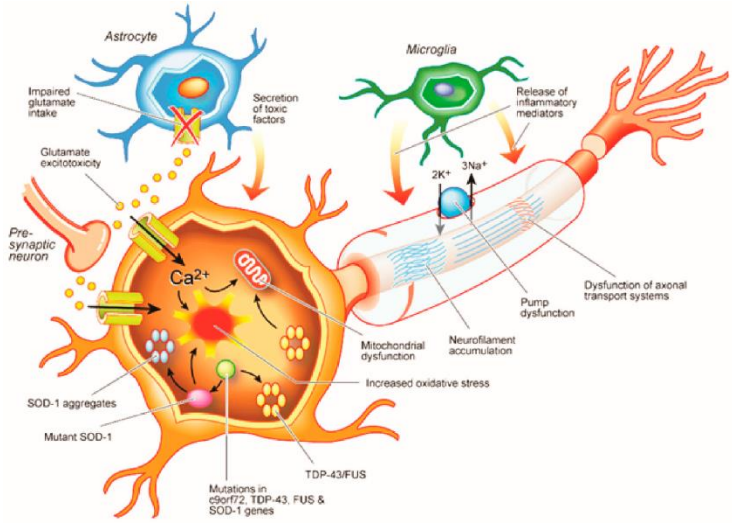


Figure 1. Overview of proposed neurotoxic mechanisms and principal mutated genes in ALS. (Modified from Van De Bos et al., Int. J. Mol. Sci., 2019)

3.2.1 Genetics

Mutations in more than 22 genes have been described in patients with ALS or ALS-like phenotypes, with more than half of these showing clear moderate or high-penetrance causative genes. These mutations can also explain some cases of sALS and some patients have more than a single gene mutated [21]. Mutations involved in ALS are sketched in Figure 2.

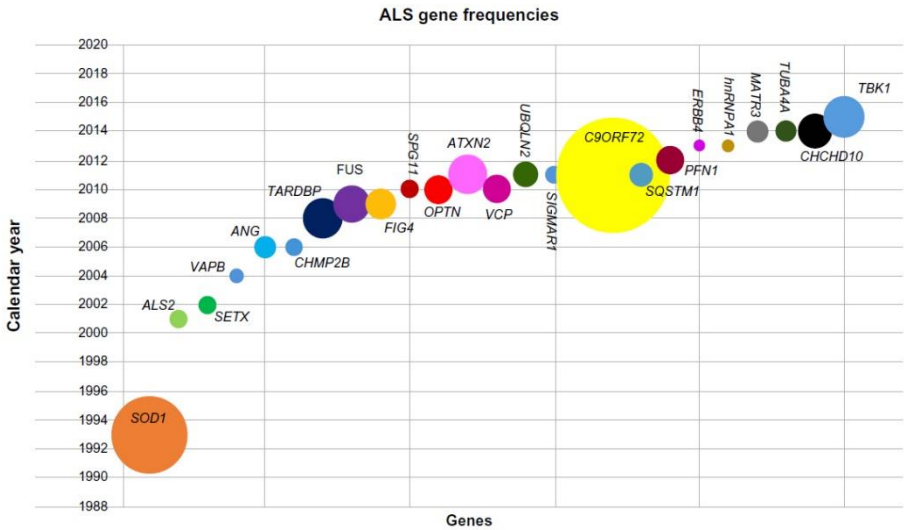


Figure 2. Genetic causes of amyotrophic lateral sclerosis. (Modified from Al Sultan A. et al., Degener Neurol Neuromuscul Dis., 2016)

SOD1. SOD1 was the first causative gene identified in ALS [22]. Located on chromosome 21, it is inherited mostly in an autosomal dominant pattern. It encodes for Cu/Zn superoxide dismutase, a protein of 153 amino acids. By now more than 160 mutations in SOD1 are associated to the disease, affecting both introns and exons. Mutations in SOD1 occur in about 15-20% of fALS [23]. Although SOD1 is the most studied gene involved in ALS, its pathogenic mechanism is still unknown. It is widely accepted that misfolded SOD1 causes mitochondrial impairment, oxidative stress and excitotoxicity [24]. SOD1-associated ALS is clinically heterogeneous, while cognitive impairment and FTD occurs rarely in these patients. Usually the clinical picture is dominated by lower motor neurons degeneration [25].

TARDBP. Mutation in TARDBP was found in 2006 due to the presence of TDP-43 (the protein encoded by TARDBP gene) in heavily ubiquitinated neuronal cytoplasmic inclusions [26]. TDP-43 is a binding protein with broad roles in RNA metabolism [27], including microRNA biogenesis [28]. Usually pathogenic mutations, affecting the C-terminal glycine-rich domain, result in the translocation of TDP-43 from the nucleus to the cytoplasm, where it forms the hallmark aggregates [29]. Mutation of TARDBP causes just 3-5% of fALS and <1% of sALS [23]. Patients with TARDBP mutation have an earlier onset of the disease and it involves usually upper motor neurons [30].

FUS. In 2009, FUS mutation was found in about 1-8% of Caucasian and in higher frequency (about 10-13%) in Asian populations. Surprisingly, a similar trend is recognized in sALS patients too. Usually it has an autosomal dominant transmission, but cases of recessive inheritance were also found [29]. Like TARDBP, FUS protein shows mutation in C-terminal domain which causes translocation in cytoplasm. In cytoplasmic compartments, FUS has a toxic gain-of-function that leads to aggregation and to the formation of stress granules. The lack of FUS in the nucleus causes impairment of RNA metabolism, including alternative splicing and gene expression regulation. Patients with FUS mutation usually show lower motor neurons symptom, early onset and shorter survival [31] [13].

C9ORF72. Mutation in C9ORF72 is the last mutation discovered, but explains about 40% of fALS and 6% of sALS, being the most common mutation [32]. Normal individuals carry 2–30 of the GGGGCC repeat units in this gene, pathological expansions are at least 700–2400 repeat units in length [29]. So far, it is not clear how an expansion in a non-coding site can cause neurodegeneration of motor neurons. Recent studies in the field have suggested different functional defects in cells related with expansion in C9orf72such as nucleocytoplasmic transport disruption, membrane-less organelle defects and DNA damage [33]. Patients with expansion often show ALS associated with FTD, early onset and short survival.

3.2.2 Oxidative stress

Mitochondrial electron transport chain is essential to produce energy in eukaryotic cells, but the use of oxygen lead to the generation of dangerous compounds, as Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS) [34]. These molecules are necessaries for many physiological processes such as signal transduction and gene transcription, however due to the unpaired electron in their outer orbit they can affect lipids, proteins and nucleic acid, and generate many by-products as peroxides, alcohols, aldehydes and ketones, that can be toxic in cells [35]. To avoid their toxicity, some antioxidant molecules (for example glutathione, vitamin E and C) and antioxidant enzymes as superoxide dismutase, glutathione reductase and peroxidase are needed. When the homeostasis between pro-oxidant and antioxidant is imbalanced, oxidative stress arises. Neuronal tissue is particularly sensitive to ROS for many reasons: presence of neurotransmitter that are an additional source of oxidative stress, glial cells that require high concentrations of oxygen to continuously generate ATP [36], presence of unsaturated lipids in neuron plasmatic membrane that are very vulnerable to oxidative stress [37] and finally, ROS can cause excitotoxic effect dysregulating intercellular calcium signaling which, in turn, promotes glutamate receptors activation [38].

In ALS, the antioxidant enzyme SOD1 becomes toxic and pro-oxidant because of the mutations that induce loss of Cu binding sites activity leading to the protein's gain of toxic activity. This stress leads to the inhibition of neurotransmitter release in neuromuscular junctions amplifying presynaptic degeneration [39]. Oxidative stress induced by SOD1 alteration is more relevant in sALS than in fALS [40].

3.2.3 Protein aggregation

Misfolded and abnormal proteins are usually present in ALS, as in other neurodegenerative diseases, and they easily tend to form aggregates and to accumulate within neurons [41]. Intracellular inclusions are usually considered an hallmark of ALS, but it is still unclear if aggregate formation directly causes neuronal toxicity, if it is only an innocuous consequence of the neurodegenerative processes or if it is a defense mechanism aimed to reduce the concentration of toxic proteins [42].

In fALS, gene mutations cause protein misfolding and accumulation, whereas in sALS it depends on aberrant post-translational modifications [43]. In motor neurons of sporadic cases protein aggregates are called Bunina bodies. They are an electron-dense amorphous material which contains vesicular or tubules vesicular structures and are positive to cystatin C12 and transferrin [44]. They are localized within the cytoplasm and dendrites, but not in the axoplasm [44]. Skein-like and roundhyaline inclusions are present too in sALS, they are placed in the cytoplasm and contain abnormal filaments (from 15 to 20 nm) that can form bundles. They are positive to

ubiquitin but negative to phosphorylated neurofilament protein and SOD1 [45]. Bunina bodies seem to be not present in familiar cases, whereas in some patients Lewy body-like hyaline inclusions were found [46].

Cytoplasmatic proteins are degraded in the cells by two main mechanisms: the ubiquitin proteasome system (UPS) for short-lived proteins [47] and autophagy for long-lived proteins [48]. In UPS, proteins that have to be degraded are tagged with ubiquitin tail that consents the degradation by proteasome [41]. In autophagy the fusion between an endosome and an autophagosome forms an autophagolysosome, which contains hydrolases that degrade proteins and organelles [49]. Normally this system permits the removal of aberrant proteins but, in this case, it does not happen and proteins precipitate. When there is a dysfunction in UPS high concentrations of ubiquitin are observed in cellular aggregates. The main component in ubiquitinated inclusions is mislocalized TDP-43 [50]. Wild-type and mutated FUS/TLS is ordered in aggregates, but it is not clear if the proteins colocalizes with ubiquitin [51] [52]. Several ALS-linked genes, including SOD1, p62, TDP43 and optineurin, participates alto to the defects of autophagy at various stages [53].

3.2.4 Excitotoxicity

In neurodegeneration often glutamate receptors are overstimulated causing excitotoxicity. This abnormal activation causes pathophysiological changes in intracellular calcium concentrations, pH, protein phosphorylation, energy metabolism and mitochondrial function [54]. An excessive cytoplasmic calcium concentration leads to the activation of Ca2+-sensitive proteases, phospholipases, protein kinases/phosphatases and NOS, causing the opening of mitochondrial membrane transition pore, osmotic swelling and rupture of outer mitochondrial membrane [55]. In ALS, reduced levels of synaptosomal high-affinity glutamate uptake and astroglial glutamate transporter EAAT2 cause the increase of extracellular concentrations of glutamate in motor cortex and spinal cord [56]. Moreover, motor neurons have a lower expression of GluR2 subunit and the reduction of post-transcriptional editing of GluR2 resulting in higher percentage of calcium permeable AMPA receptors, causing a greater sensitivity to excitotoxicity [57]. In ALS mouse models, morphological features of excitotoxicity in motor neurons include somatodendritic swelling, mitochondrial damage and chromatin condensation,that are typical of cells during necrosis. However, in other kind of neurons, excitotoxic process is accompanied by cytological features apoptosis-like [54]. Altered glutamate handling is a common characteristic between sALS and fALS and could be used to develop pharmacological treatments. Drugs increasing EAAT2 activity, for instance, extend survival [58] and riluzole, decreasing glutamate release, slows the disease course only of few months [59].

3.2.5 Mitochondrial impairment

In motor neurons of ALS patients, axonal mitochondria have peculiar morphological features which consist in rounding up [60], fragmentation of the networks [61] and swelling of internal cristae [61]. Besides the structural abnormalities, pathology concerns mitochondrial functional defects, such as alterations of oxidative phosphorylation chain and mutations in mitochondrial DNA [62]. This malfunctioning can cause oxidative stress and increasing of ROS generation that induces high damage to the mitochondrion itself and to the entire cell [62]. On the other hand, higher levels of superoxide have been linked to more frequent mitochondrial branching [63] that causes the apoptotic cascade's initiation [64].

Motor neurons, compared to other cells, express lower levels of cytosolic calcium buffers, as parvalbumin [65], so the mitochondria have the fundamental role of calcium buffering. When they are unable to maintain ion's homeostasis, cytoplasmic calcium level increases and the mitochondria membrane potential decreases [66], favoring the abolition of the fusion process and the formation of membrane permeability pore [64]. Moreover, high intracellular calcium concentration interferes with mitochondria movement toward the extremities of long axons causing transport system failure [67].

It is still unclear if mitochondrial dysfunction may be considered as either a cause or a consequence of neurodegeneration, even if studies in motor neurons disease rodent models indicate that mitochondrial abnormalities begin prior to the clinical and pathological onset of the disorder, suggesting a causal role of mitochondrial dysfunction [62]. Giver their central role, different therapeutic strategies focusing on mitochondria have been tested to stop or slow down ALS progression, but neither treatments aimed at increasing mitochondrial function and survival nor those aimed at reducing oxidative stress have yielded significant results in clinical trials, despite promising results in animal models [68].

3.2.6 Axonal transport dysfunction

Axonal transport mediates the movement of cargoes such as proteins, mRNA, lipids, membrane-bound vesicles and organelles that are mostly synthesized in the cell body to their correct spatiotemporal distribution in the axon. Moreover, axonal transport maintains the essential long-distance communication between the cell body and synaptic terminals allowing neurons to react to their surroundings via trafficking of different molecules. In both sALS and fALS patients, axonal transport defects are a common observation and, moreover, the impairment of slow axonal transport is one of the earliest pathological events in ALS [69]. Beside the abnormal accumulations of phosphorylated neurofilaments, an hallmark of ALS, different mutations in components of the slow and fast axonal transport machinery, such as kinesin and dynein, have unequivocally been correlated to neurodegeneration [70]. Detailed

analysis of axonal transport revealed defective dynein-mediated retrograde transport in the intact sciatic nerve of presymptomatic SOD1G93A transgenic mice [71]. Furthermore, also mutations in alsin (ALS2) and charged multivesicular body protein 2B (CHMP2B) have been detected; they cause neurodegeneration disrupting the retrograde axonal transport of endosome [72] [73].

Mitochondrial axonal transport is also impaired in ALS. In motor neurons of early symptomatic SOD1G85R and SOD1G37R transgenic mice, the number of axonal mitochondria is reduced and their distribution is no longer homogeneous throughout the axon [74]. Moreover, it has been demonstrated that, in SOD1 mutated neurons, mitochondrial transport is impaired in both anterograde and retrograde directions [67], resulting in abnormal mitochondrial morphology, fewer organelles in the axon, reduced energy production and decreased calcium buffering capacity [75].

3.2.7 Contribution of non-neuronal cells in inflammatory process

The main cause of ALS is motor neurons degeneration, however it has been demonstrated that toxicity is non-cell autonomous, indicating that damage develops within cell types beyond neurons themselves [76]. In neurodegeneration also microglial cells are plenty involved, due to their ability to release trophic and toxic molecules. They were found activated in the brain and spinal cord of ALS patients before motor neuron loss [77]. Microglial cells have also a macrophagic role in removing axonal degeneration's debris. These cells stimulate the production of pro-inflammatory cytokines, such as IFN-γ and TNFα, expressing cyclooxygenase-2 (COX-2) [78]. In physiological condition, pro-inflammatory molecules activate immunoproteasome expression, which helps neurons to rapidly degrade oxidize proteins. However, cytokines cause inflammation that results in ROS production and excitotoxicity [79]. Furthermore, immune system is involved in ALS inflammatory response since regulatory T cells lower microglia neuroinflammation releasing anti-inflammatory cytokines [80]. From the early stages proceeding to the end-stages of the disease, the immune response shift from a neuroprotective role to a cytotoxic one: at the beginning T cell population in spinal cord is mainly composed of helper CD4+ cells, then cytotoxic CD8+ increases rapidly and become the main T-cells type [40]. For all these reasons, ALS must be considered not only a motor neuron disease but also a pathology involving neuronal neighboring cells.

3.2.8 Cell cycle and apoptosis

There is growing evidence for involvement of cell cycle regulators genes in neurodegenerative disorders and in apoptotic death of neurons subjected to various insults. Cell-cycle proteins and transcriptional regulators such as cyclins, cyclin-associated kinases, the retinoblastoma gene product (pRb), and E2F-1 function during

cellular proliferation, differentiation, and cell death pathways. It has been demonstrated that G1 to S phase activation occurs during ALS caused by hyperphosphorylation of the retinoblastoma protein, increased levels of cyclin D and redistribution of E2F-1 into the cytoplasm of spinal cord motor neurons and glia [81]. Moreover, in spinal cord of ALS there are elevated levels of p53 and an increase in Bax, Fas, caspase-8 and -3 proteins, linking p53 and activation of G1 to S phase to motor neurons cell death [82].

Cell-cycle alteration has been observed also in SOD1 mutated mouse model. In particular, in SOD1G37R mice has been demonstrated an upregulation and mislocalization of Cdk4, a regulator of the G1-S checkpoint. This increase was associated with an increase in nuclear Cdk4, cyclin D1, its coactivator, and with the abnormal phosphorylation of the retinoblastoma protein at Cdk phosphorylation sites. These results indicate that a cell cycle signaling at the neuronal G1-S checkpoint may constitute a critical step of the neuronal death pathway in ALS caused by mutant SOD1 [83]. Another study demonstrated that SOD1 mutant G93A led to a decreased expression of Spy1, a member of the cell cycle regulator Speedy/Ringo family that enhances cell proliferation and promotes tumorigenesis. Decreased Spy1 expression reduces cell viability and further activates the DNA damage response in mSOD1 cells, while its overexpression improves cell viability and inhibits DNA damage response. These results suggest that Spy1 plays a protective role in ALS motor neurons and moreover, provide novel direction, such as investigating the role of oncogenic proteins in ALS [84].

3.3 Transcription alteration in ALS

It has become increasingly clear that transcriptional dysregulation is a key contributor to ALS pathogenesis. The major ALS genes SOD1, TARDBP, FUS, and C9orf72 are involved in every aspects of RNA metabolism processes, making coding and non coding RNA deregulation extremely important in the pathogenesis of ALS.

3.3.1 RNA metabolism in ALS

Perturbations in RNA processing have been described in ALS at various levels such as gene transcription, alternative splicing, RNA transport, mRNA stabilization and miRNA biogenesis [85]. Recently, changes in gene expression in ALS patients have been demonstrated [86] and the discoveries of mutations in key RNA binding proteins involved in ALS have placed the RNA metabolism as a central process to disease etiology [51] [87]. As a matter of fact, mutations in different DNA/RNA-binding proteins, as causes of both fALS and sALS, has opened enormous perspectives on the implication of post-transcriptional mechanisms alterations in ALS pathogenesis.

Alterations in gene expression have been documented in different tissues from ALS patients using microarray and RNA-seq analysis.

TDP43. TARDBP mRNA gene is upregulated by 1.5–1.8 fold-change in Peripheral Blood Mononuclear Cells (PBMCs) from patients affected by ALS disease [88]. Moreover, TDP- 43 mRNA is abnormally upregulated in the spinal cord of ALS subjects causing neuronal death by increasing of microglia neurotoxicity [89]. Concerning the TDP-43 protein, it regulates the splicing patterns of transcripts of several important genes, TARDBP itself, FUS, HTT (Huntingtin), SNCA (α-synuclein) and APP (Amyloid precursor protein) etc. [90] [91]. Nuclear depletion, over-abundance and mutations in TDP-43 results in mRNA splicing aberrations which is detrimental to neurons [92] [93] [94]. Furthermore, TDP-43 associates with RNA molecules to form ribonucleoprotein (RNP) granules which transport mRNA to distant locations. In ALS mutants TDP-43 were found to impair the transportation of the RNP granules [95]. TDP-43 regulates stabilities of several mRNAs, including that of its own mRNA [96], interacting with the regulatory 3' UTR sequences of these mRNA and affecting their half-like, both positively, as the human low molecular weight neurofilament mRNA, or negatively, as documented for the vascular endothelial growth factor and progranulin mRNA transcripts [96] [97]. TDP-43 can also affect the translation of several mRNAs via sequestration of the translation factors into stress granules [98] and forming complexes with other proteins involved in the translation machinery [99].

FUS. Fused in sarcoma and translocated in lipoSarcoma (FUS/TLS) is a heterogeneous ribonucleoprotein (hnRNP) that, as TDP-43, is involved in RNA splicing, transportation, and stabilization [100]. Its mRNA levels are not deregulated, but mutations in FUS might impact target gene expression [101]. Indeed, it has been demonstrated that FUS regulates the alternative splicing of genes crucial for neurogenesis and gene expression regulation. FUS alternative splice targets in mouse brain, such as Ntng1, Ndrg2, Nrxn1, Nlgn1 and Camk2a, play crucial roles in axonogenesis, cytoskeleton organization, cell adhesion, and synaptic formation and function [102]. The dysregulation of these RNA targets may cause synaptic dysfunction, axon withdrawal and denervation, which leads to neurodegeneration in ALS and FTD. Although thousands of FUS RNA targets have been identified, only few targets have been characterized to elucidate how dysregulated alternative splicing of specific RNAs can contribute to ALS pathogenesis. For example, exon 10 of the microtubule-associated protein Mapt/Tau is a known target of FUS. Knockdown of FUS in rat hippocampal neurons promotes Mapt/Tau exon 10 inclusion and consequently causes cytoskeleton disorganization, shortened axons and enlarged growth cones that may lead to neurodegeneration [103].

SOD1. Increased SOD1 mRNA expression in spinal cord, brain stem and lymphocytes of sALS patients have been reported [104]. Although SOD1 protein does not contain canonical RNA binding motifs, mutSOD1, due to conformational changes, acquires new toxic functions by exerting direct RNA binding activities and consequently altering the turnover of the target mRNAs [105]. It has been shown that

the presence of mutSOD1 impairs a network of RNA-binding proteins (RBPs) and causing the destabilization of mRNA species, such as hNFL [106] and vascular endothelial growth factor (VEGF) mRNAs [105]. The destabilizing effects on VEGF mRNAs is mediated directly by SOD1 binding to specific adenylate/uridylate-rich elements (AREs), located in 3'-UTR of transcripts [105]. These cis-acting elements are specifically recognized by different factors, including the embryonic lethal abnormal visual (ELAV) family of RNA-binding protein, composed of 3 neuron specific members (HuB, HuC, and HuD) and 1 (HuR or HuA) ubiquitously expressed. It has been demonstrated that mutSOD1 competes with HuR and HuC proteins, thus impairing the post-transcriptional processing of different mRNA, such as VEGF and potentially other ELAVs targets [107]. Finally, SOD1 can act also as a transcription factor binding to promoters and regulating the expression of oxidative resistance and repair genes [108].

C9orf72. C9orf72 expansion repeat can interfere with transcription and splicing or C9orf72 transcripts itself [109]. It has also been proposed that the C9orf72 expansion repeat could disrupt its promoter activity thus reducing its expression [110]. Several studies have reported alterations in the C9orf72 transcriptome, recently an article showed an increased expression of the calcium-permeable GluA1 AMPA receptor subunit in motor neurons derived from IPSC of C9orf72 mutated patients [111]. AMPA reduction led to an enhanced motor neurons vulnerability to excitotoxicity [111]. Additionally, it has been established in other neurodegenerative diseases, such as Fragile X-associated Tremor/Ataxia Syndrome (FXTAS), that expanded RNA forms pathogenic foci that trap one or more RNA binding protein(s) resulting in their depletion and loss of function [112].

3.3.2 Non coding RNAs

Much of the non-protein-coding part of the human genome has historically been regarded as junk DNA. However, over the last decade, the development of high-throughput technologies, such as Next Generation Sequencing (NGS), has allowed an in-depth examination of the non coding genome with unprecedented resolution and scale. These studies have surprisingly revealed that, although less than 2% of the human genome encodes proteins, the majority of all nucleotides are detectably transcribed under some conditions [113]. The potential function of ncRNAs is still enigmatic, although functional ncRNAs are identified as regulatory molecules and play roles in multiple biological processes. NcRNAs classification is still in progress. Regarding the expression of ncRNAs, they can be divided into housekeeping ncRNAs and regulatory ncRNAs. Housekeeping ncRNAs are constitutively expressed and include transfer, ribosomal, small nuclear and small nucleolar RNAs. Regulatory ncRNAs are further divided into two classes based on nucleotide length (Figure 3). Those less than 200 nucleotides are usually referred to as short/small ncRNAs and include microRNAs (miRNAs), that have been already identified as crucial regulators of gene expression in multiple physiological and pathological

mechanisms [114]. LncRNAs are linear or circular transcripts longer than 200 nucleotides and they have been object of more recent study because of the several evidences suggesting their involvement in fundamental cellular processes.

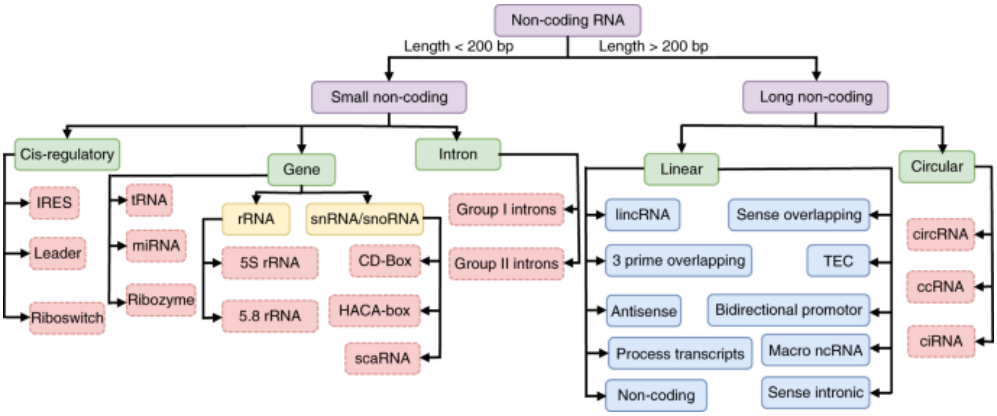


Figure 3. Non coding RNA classification overview. (Modified from Noorul Amin et al., Nature Machine Intelligence, 2019)

3.3.3 miRNAs

MiRNAs are small, regulatory RNAs that control the translation of protein coding RNAs. MiRNAs directly perform translational repression by partial binding to the 3′ UTR of mRNAs as a complex, the RNA-induced silencing complex, with Argonaute-2 (Ago2) [114]. Since only partial complementarity is required for miRNA–mRNA interactions, a single miRNA can potentially regulate hundreds of mRNAs. Multiple studies demonstrated that miRNAs are powerful regulators of physiological and pathological cellular processes. MiRNA expression is often dysregulated in disease and, thus, they have been used as both therapeutic and diagnostic targets [115]. The miRNAs biogenesis is showed in Figure 4.

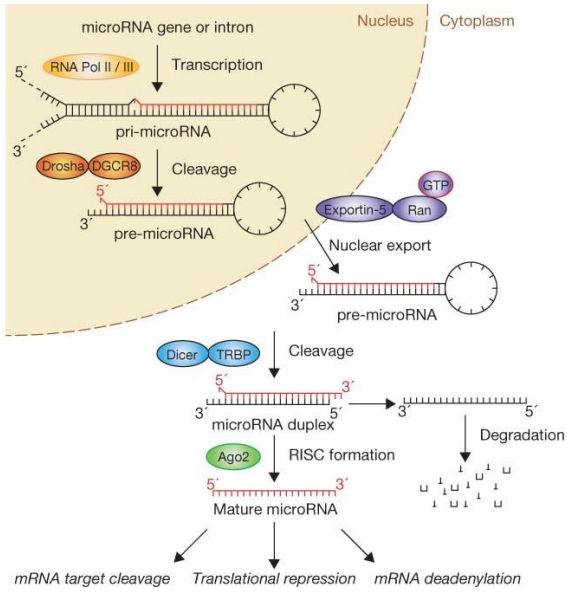


Figure 4. The 'linear' canonical pathway of microRNA processing. (Modified from Winter et al., Nature Cell Biology, 2009)

3.3.4 Role of miRNAs in pathogenesis of neurodegeneration

Approximately the 70% of miRNAs is expressed in the nervous system [116] and there is evidence that altered activity of miRNAs can be linked to neurodegenerative diseases [117]. In ALS, miRNAs have been investigated to find out new possible pathological mechanisms and to use them as early biomarkers. Indeed, in sALS, a particular set of 8 miRNAs are deregulated in blood leukocytes, representing potentially useful biomarkers of early-stages ALS [118]. For example, miR-338-30 has been found upregulated in blood, serum, cerebrospinal fluid (CSF) and spinal cord tissue of sALS patients [119]. In addition, also in fALS patients, the expression of a set of miRNAs has been identified in the serum as a pathological molecular signature [120]. Interestingly, 24 of those miRNAs appear to be down-regulated in asymptomatic mutation carries up to 20 years before disease the estimated time window of disease onset [120]. Aberrant regulation of miRNAs may be also involved in the pathogenesis of ALS. They participate to all the altered processes in ALS. For example, overexpression of miR-23a downregulates the skeletal muscle peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1 α), an important protein for normal mitochondrial function [121]; by contrast, down-regulation of miR-2403 and miRb1336 may influence the expression levels of the neurofilament light chain, impacting on axonal transport [122]. These observations also suggest that the manipulation of miRNA levels could be a promising tool for ALS intervention.

Interestingly, TDP-43 and FUS play role in miRNA biogenesis (Figure 6). For instance, knockdown of TDP-43 has been shown to upregulate miRNA let-7b and downregulate miR-663 [123]. Moreover, both nuclear and cytoplasmic TDP-43 can regulate miRNA expression [124], as seen for the miR-206 and miR-1 families, although the significance for ALS etiology remains unclear [125]. FUS controls miRNA expression by interacting with DROSHA [126]. In addition, in physiological condition FUS upregulates the expression of miR-200a and miR-141 with binding sites in 3' UTR. The G48A FUS mutation in 3' UTR abolishes the miRNA binding site, thus disrupting their regulation [127]. These data suggest that mutations and alterations in TDP-43 and FUS may trigger abnormal RNA expression, thereby bearing on ALS pathogenesis.

3.3.5 Long non-coding RNAs

The lncRNAs are defined as non coding RNA molecules longer than 200 nucleotides. Most of them are transcribed by RNA polymerase II, thus sharing similarities mRNAs, such as the 5′ 7-methylguanosine cap and a 3′ poly(A) tail, however, they lack coding capacity [128]. So far, the ENCODE project (GENCODE v26) has conservatively annotated in humans close to 16,000 lncRNA genes that give rise to more than 28,000 distinct transcripts. Additionally, protein-coding genes too can produce transcript variants that lack coding capacity, adding to the vast catalogue of long non coding transcripts present in the cells. Despite not being translated into proteins, lncRNAs are functional molecules with high heterogeneity and functional versality that relies on their ability as long RNA molecule to conform to different structures and molecular interactions. Indeed, lncRNAs can regulate, among the others, transcriptional regulation in cis or trans, organization of nuclear domains, and regulation of proteins or RNA molecules [129]. Moreover, the deregulation of lncRNAs has been related to different human diseases, including cancer, cardiovascular and neurodegenerative diseases [130]. LncRNAs can regulate gene expression by different mechanisms, some of which are illustrated in Figure 5. These modes of action include lncRNA transcription-dependent activation or repression of neighbor genes (1), lncRNA-mediated inter-chromosomal interactions (2), formation of nuclear structures (i.e. paraspeckles) (3) or R-loops (4), lncRNAs as guide (5) or decoy (6) of transcription factors or as a scaffold for chromatin modifying complexes (7), lncRNAs acting as sponges of miRNAs (8), regulating post-transcriptional mRNA decay (9), regulating the cellular localization of RNA-binding proteins (RBPs) (10) or DNA-binding proteins (DBPs) (11).

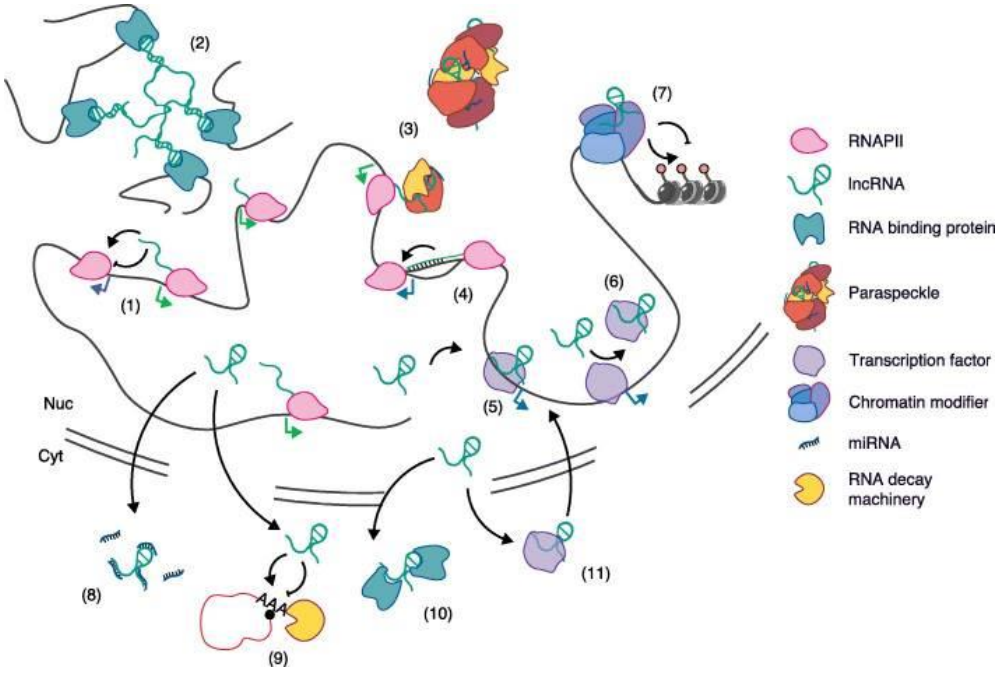


Figure 5. LncRNAs functions. (Modified from Marchese et al., Genome Biol., 2017)

Inside the nucleus, lncRNAs regulate gene expression through different mechanisms that can be inherent to the RNA molecule or linked to its gene locus. There are at least three potential mechanisms through which a lncRNA locus can locally regulate chromatin or gene expression (Figure 6): (1) the lncRNA transcript itself regulates the expression of neighboring genes through its ability to recruit regulatory factors to the locus and/or modulate their function, (2) the process of transcription and/or splicing of the lncRNA confers a gene-regulation functionality that is independent of the sequence of the RNA transcript, or (3) regulation in cis depends solely on DNA elements within the lncRNA promoter or gene locus and is completely independent of the encoded RNA or its production.

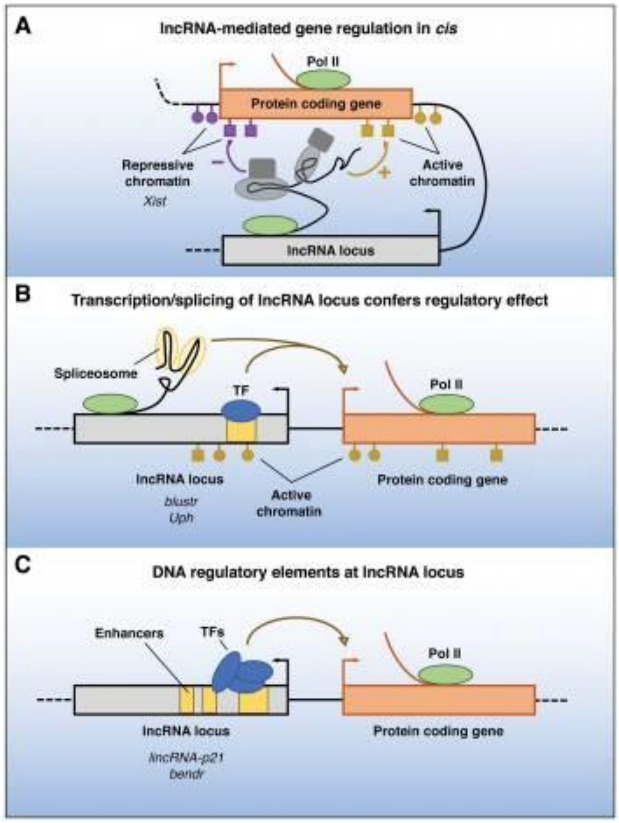


Figure 6. Functions of lncRNA loci in local gene regulation. (Modified from Kopp et al., Cell, 2018)

Moreover, there is an increasing number of examples of lncRNAs that leave the site of transcription and operate in trans. These lncRNAs can be categorized into at least three major subgroups (Figure 7): (1) lncRNAs that regulate chromatin states and gene expression at regions distant from their transcription site, (2) lncRNAs that influence nuclear structure and organization, and (3) lncRNAs that interact with and regulate the behavior of proteins and/or other RNA molecules, for example with the mechanism of competing endogenous RNA (ceRNA) through which lncRNAs regulate the abundance or activity of other RNAs (usually miRNAs) to which they bind through base-pairing interactions [131].

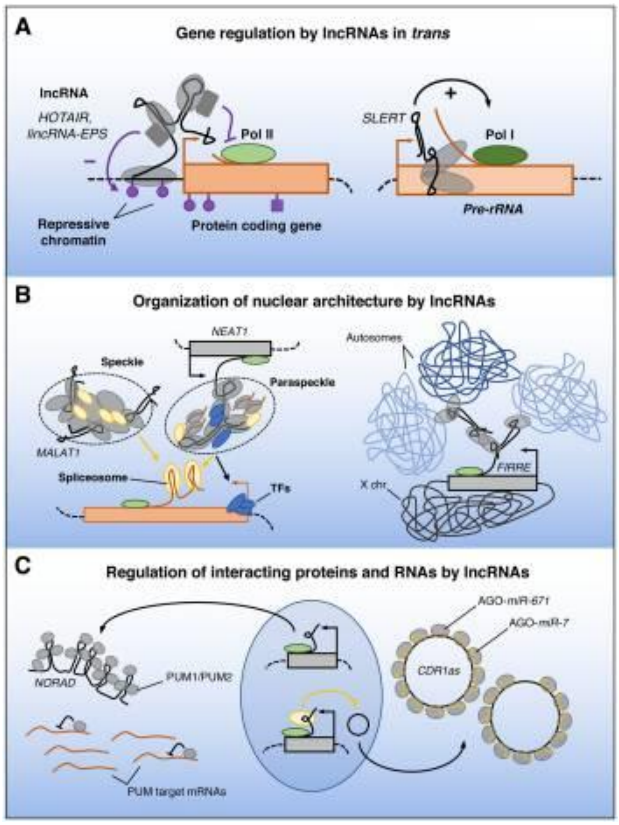


Figure 7. Functions of lncRNAs in trans. (Modified from Kopp et al., Cell, 2018)

Despite the rapid growth of the field, intriguing questions remain, such as whether all or just a fraction of the existing lncRNAs has a function, or whether this function can be exclusively ascribed to the RNA product of the lncRNA gene.

3.3.6 Role of lncRNAs in pathogenesis of neurodegeneration

LncRNAs are highly expressed in the nervous system [132] and their networks are highly adapted to complex neurobiological functions. The roles of lncRNAs in brain development, neuron function, maintenance, and differentiation, as well as neurodegenerative diseases, are becoming increasingly evident. Indeed, recent studies have shown that lncRNAs have a significant impact on normal neural development and on the onset and progression of neurodegenerative diseases, including Alzheimer's disease (AD), Parkinson's disease (PD), FTD and ALS. The mechanisms of action some lncRNAs are providing new insights into our understanding of the etiology and pathophysiology of the neurodegenerative diseases.

One of the best-known lncRNA that regulates a key gene in human neurological disease is β-secretase-1 antisense RNA (BACE1-AS) in AD. One of the main pathological changes associated to AD is the extracellular aggregation of amyloid plaques derived from the proteolytic processing of the amyloid precursor protein (APP) by the β-site amyloid precursor protein-cleaving enzyme (BACE1). In 2008 a conserved antisense transcript, BACE1-AS, has been identified at the BACE1 locus [133]. BACE1-AS upregulates BACE1 mRNA by forming stabilizing duplex with the mRNA leading to an increase in BACE1 protein levels [134]. In contrast, the Aβ-stimulated upregulation of NAT-Rad18, the antisense transcript of the DNA damage repair gene Rad18, leads to a reduce of Rad18 protein in AD, causing DNA damage stress and neuron apoptosis [135].

In PD, two antisense RNAs of two well-known PD genes have been described. PINK1-AS is transcribed from the antisense orientation of the PTEN-induced kinase 1 (PINK1) and it can stabilize the expression of PINK1, whose dysregulation leads to impairment in dopamine release and motor deficits [136]. UCHL1-AS, identified as antisense transcript of the ubiquitin carboxy-terminal hydrolase L1 (UCHL1), promotes the association of its sense protein coding mRNA with active polysome in cytoplasm for translation. This mechanism is disrupted in some cases of familial PD, and the loss of UCHL1 activity has been reported in many neurodegenerative disease [137].

Even less information is available about the role of lncRNAs in FTD. TDP-43 and FUS bind non coding RNAs and affect their transcription and activity [138]. Most of the information regards the non coding transcripts identified at the C9orf72 locus, shared with ALS. The C9orf72 repeat expansion region undergoes bidirectional transcription [139]. Antisense C9orf72 transcripts are upregulated in the brains of both FTD and ALS patients where they form nuclear RNA foci [140].

3.3.7 Long non coding RNAs and ALS

The involvement of abnormalities in functional RNAs has been also reported in the development of ALS. Recent genome-wide analyses of the human transcriptome have revealed a plethora of lncRNAs whose biogenesis, regulation, and cellular roles are only starting to be elucidated. Increasing evidences may address the deregulation of lncRNAs as key modulators of ALS pathogenesis.

The complexity of ALS disease is due to the numerous different cellular features, one of those are the “paraspeckles”, nuclear bodies formed by a set of specialized proteins and RNAs, such as the lncRNA Nuclear-Enriched Abundant Transcript 1_2 (NEAT1_2T) [141], that was identified associated to ALS in brain [142]. It has been shown that TDP-43 and FUS/TLS were enriched in paraspeckles and bound directly to the transcript [142]. Indeed, both TDP-43 and FUS/TLS are paraspeckle proteins, which are thought to be required for normal paraspeckle formation through the direct

interactions with NEAT1_2 lncRNA [141]. The frequency of paraspeckle formation is highly increased during the early phase of ALS pathological course, thus it has been concluded that NEAT1_2 could act a scaffold of RNA binding proteins in the nuclei of ALS motor neurons [141].

Ataxin 2 (ATXN2) is a coding gene recently linked to ALS by the association between the length of ATXN2 repeat expansion and risk of ALS [143]. Recently, it has been demonstrated that ATXN2 locus is bidirectionally transcribed in ALS tissues with ATXN2 expansion [144], giving rise to the antisense transcript ATXN2-AS. The antisense transcript ATXN2-AS with a CUG repeat expansion is neurotoxic, and therefore may contribute to ALS pathogenesis, forming CUG repeats hairpin structures that shows sponge-like properties and sequester RNA-binding proteins [145].

The expansion of C9orf72 is also expressed in the antisense direction and provide evidence that antisense RNA foci contribute to C9orf72 ALS/FTD [146]. More than one toxicity mechanism has been linked to C9orf72-AS. Indeed, cellular toxicity has been proposed to result either from a gain of function or from a loss of function of the C9orf72 gene [147]. RNA toxicity is led by the hexanucleotide repeats that are transcribed in both sense and antisense directions, resulting in nuclear aggregates of sense and antisense RNA. Moreover, C9orf72 repeat RNA forms secondary structures such as hairpins and G-quadruplexes, leading to the formation of intranuclear RNA foci, which are generally assumed to become toxic by the sequestration of RNA-binding proteins [148]. Finally, toxicity derived from dipeptide proteins translated from the repeat RNA through repeat-associated non-ATG initiated translation (RANT) has been observed in ALS mouse model [149].

3.3.8 Transcriptional factors alterations in ALS

Due to the importance of RNA metabolism and expression in the pathogenesis of ALS, many studies have focused their attention on the role of dysregulated transcription factors. Phenotypic changes effected by pathological events are now routinely captured by gene expression profile measurements, determining mRNA abundance on a genome-wide scale in a cellular population. Following this approach, Ikiz and collaborators [150] performed computational assembly and interrogation of neural tissue specific interactomes of purified embryonic stem cell-derived motor neurons after exposure to mutSOD1 astrocyte conditioned medium. They identified 23 transcription factors as candidate drivers of neurodegeneration. 14 of those (~64%) were shown to modulate motor neurons loss, including a key subunit of the nuclear factor kappa B (NF-κB) complex, whose role as effector of mutSOD1 astrocyte-induced motor neurons death was further confirmed by genetic and pharmacological approaches [150].

Another inflammation-involved transcription factor, Nurr1, has been found dysregulated in ALS [151]. Indeed, Nurr1 expression levels were upregulated in the peripheral blood of ALS patients. Moreover, Nurr1 was strongly upregulated in spinal cord of SOD1-G93A mouse model during the asymptomatic and early symptomatic phases of the disease, promoting the expression of brain-derived neurotrophic factor mRNA and repressing NFkB pro-inflammatory targets. Based on these data, Nurr1 could be activated in an early phase of the disease as a protective mechanism, although not sufficient to reverse the disease progression.

Autophagy is another central pathway involved in ALS and Transcription factor EB (TFEB) was recently discovered to be a master regulator of lysosome biogenesis and autophagy [152]. As a matter of fact, TFEB increases the activity of lysosomal degradative pathways [153], stimulates endocytosis [154] and exocytosis [155] and contributes to maintain neuronal proteostasis. Interestingly, TFEB has been shown to reduce pathogenic protein accumulation in Huntington's disease cellular model [153] and in in a PD mouse model [156]. In ALS brain, nuclear TFEB levels were reduced by 62%, likely causing the widely reported autophagy defects.

Endoplasmic reticulum (ER) stress represents an early pathological event in ALS [157]. ATF4 is a key ER stress transcription factor that plays a role in both adaptation to stress and the activation of apoptosis [158]. It has been described that SOD1-G86R transgenic mice lacking ATF4 were more resistant to develop ALS with delayed disease onset and prolonged life span, attenuating the induction of pro-apoptotic genes, such as BIM and CHOP, and leading to quantitative changes in the ER protein homeostasis network [159].

Rahman and collaborators [160] through computational approach found dysregulation of 5 transcription factors in ALS patients. They analyzed blood cell and brain transcriptomics gene expression datasets, RNA-seq and microarray, and identified 13 differentially expressed genes involved in neurodegeneration-associated pathways that were commonly dysregulated between ALS blood and brain tissue. They integrated these data in order to reveal regulatory biomolecules that can control the expression of deregulated transcripts and they identified 5 deregulated transcription factors, SP1, MYC, TP53, CTCF and SRF, well-known to be involved in cancer.

Finally, in our recent work [86] we performed RNA-seq in PBMCs from sALS patients and matched controls and we found that the top differentially expressed mRNAs impact on the transcription pathway, as shown by GO enrichment analysis: transcription regulation is indeed the second most involved pathway in sALS patients. In particular, ZMYM6, TTF2 and TAF5L are associated to nucleic acid binding and transcription [161] and MYCBP is the MYC-binding protein. This association emerged also respect to molecular function: the most enriched GO terms targeted by down-regulated mRNAs include transcription factor activity, activating transcript factor binding and DNA binding. Involvements of the transcription pathway is evident also considering the most deregulated lncRNAs in sALS group. Three

lncRNAs reported in the top 10 are described as antisense of transcription-related genes: ZEB1-AS1 is indeed antisense of ZEB1 transcription factor, ZBTB11-AS1 is the antisense of ZBTB11 gene, involved in the DNA binding and in transcriptional regulation, and also XXbac-BPG252P9.10 is described as the antisense transcript of IER3, involved in transcription; one lncRNA is directly induced by the transcription factor MYC and participates to its network, MINCR.

3.4 A shared mechanism of RNA metabolism in cancer and neurodegeneration

In the last decade, different studies have proposed an association between cancer and neurodegenerative diseases, two disorders that seem to have little in common. Although the two pathologies share disruptions in a wide range of cellular pathways, including cell cycle, cell survival and cell death, the outcomes are very divergent: uncontrolled cell survival and proliferation in cancer and progressive neuronal cell death in neurodegeneration. Mutations in genes involved in DNA repair pathways, regulation of the cell cycle, protein turnover, autophagy and oxidative stress have been implicated in both of these diseases [162].

Epidemiological studies have demonstrated an interesting association between cancer and neurodegenerative diseases. For instance, a decreased risk of developing cancer has been reported among individuals with PD [163]. In a similar way, it has been shown that AD is associated with a reduced risk of cancer, while patients with cancer have lower incidence of AD [164]. In contrast, it has been reported also positive correlation between certain types of cancers and neurodegeneration. Indeed, skin carcinoma and malignant melanoma occur more frequently in PD population compared to controls [165].

Among the mechanisms affected in cancer and neurodegenerative diseases, alterations in RNA metabolism are obtaining more and more attention given the critical role for RNA transcription, maturation, transport, translation, stability and degradation in normal cellular function. For instance, there are many evidences that RBPs are the key molecular linkage between neurodegeneration and cancer. Altered expression levels, abnormal aggregation, gene mutations or translocation and loss of activity could have a great impact on the cell physiological condition, leading to uncontrolled proliferation or progressive death depending on the cellular type affected and the involved molecular alteration (Figure 8). Generally, high expression levels or gain-of-function of RBPs gene occur in cancer cells, instead low expression levels, mislocalization and cellular inclusions have been linked to neurodegenerative cells [166].

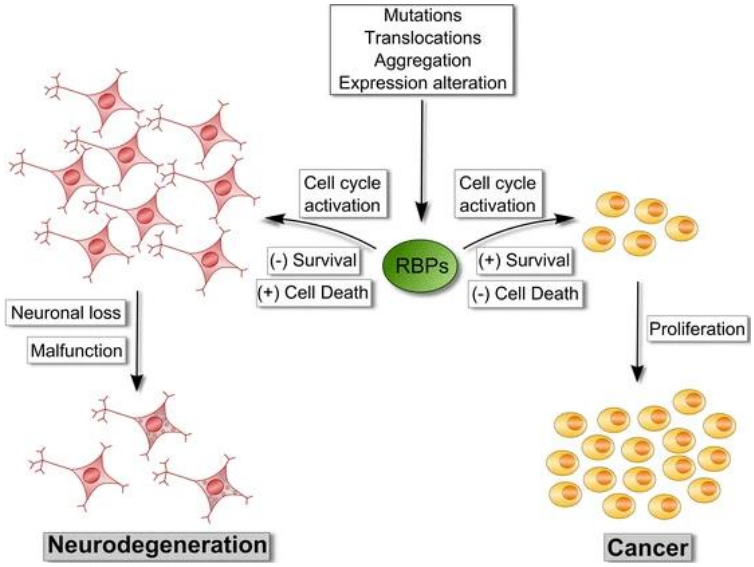


Figure 8. Alterations RBPs on the RNA metabolism could impact pro-survival and cell death mechanisms, as well as cell cycle leading to cancer or neurodegeneration depending on the molecular defect and the cell type affected. (Modified from Campos-Melo et al., Biogerontology, 2014).

Changes in microRNAs also emerged as potential regulators of both cancer and neurodegenerative pathologies [167]. In particular, individual miRNAs, such as miR-9, miR-29 and miR-34, have been implicated in the initiation and progression of both tumor and neurodegenerative conditions by regulating common pathways or targeting specific genes [168] (Figure 9).

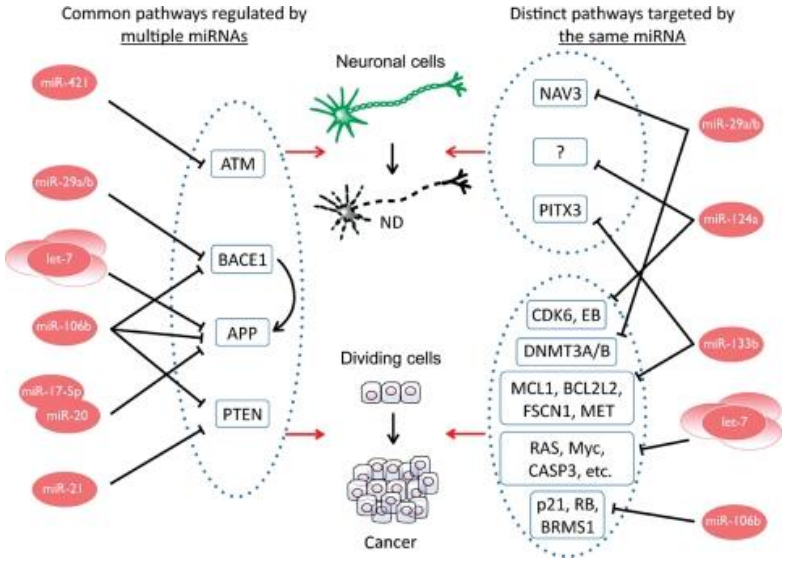


Figure 9. Summary of the common pathways underlying neurodegenerative diseases and cancer that are mediated by miRNAs. (Modified from Du et al., J Mol Cell Biol, 2011).

The correlation between cancer and neurodegeneration exists also for ALS. Indeed, epidemiological data suggest a significantly reduced risk of ALS amongst prostate cancer survivors, while an elevated risk of ALS amongst survivors of melanoma and tongue cancer has been observed [169]. The link between the two clinical phenotypes resides also in RNA metabolism. For instance, TDP-43 participates to ALS as well as to Ewing sarcoma where a variant in a region near to TARDBP gene constitutes a strong Ewing sarcoma susceptibility locus [170]. Furthermore, TDP-43 is up-regulated in hepatocellular carcinoma where it regulates the expression of the platelet isoform of phosphofructokinase (PFK) through a negative regulation of miR-520a-3p, miR-520b and miR-520e, suggesting that TDP-43 is a novel transcriptional regulator of glycolysis in cancer [171]. Also alterations in FUS/TLS expression, typical of ALS, have a controversial role in prostate cancer. On the one end, overexpression of FUS/TLS significantly retards androgen-induced prostate cancer growth both in vivo and in vitro [172]. On the other hand, it has also been reported that the reduction of FUS/TLS levels limited androgen induced proliferation of the tumour [173].

3.4.1 MYC

The MYC oncogene was one of the first human genes identified that can drive cellular transformation. It encodes the c-MYC (MYC) transcription factor and other tissue-specific family members were subsequently identified in neuroblastoma and lung cancer as MYCN (N-MYC) and MYCL (L-MYC), respectively [174]. Like most regulatory transcription factors, MYC contains two separable domains, the C-terminal and the N-terminal, both essential for the biological function of MYC, which works only in complexes. The C-terminal domain mediates DNA binding to target genes through a heterodimer complex with basic helix – loop – helix/leucine zipper (b/HLH/Z) protein Max [175]. Max, through its leucine zipper domain, can bind to itself or to other transcription factors forming homo- and hetero-dimers, respectively. When bound to DNA, the MYC-Max heterodimer recruit complexes that modify chromatin. (Figure 10). MYC N-terminal domain binds another specific protein, MYC-Binding Protein (MYCBP), which stimulates the activation of E-box-dependent transcription by MYC [176]. MYCBP is normally found in the cytoplasm, but it translocates to the nucleus during S phase of the cell cycle and associates with MYC. Both the two domains are essential for the biological function of MYC.

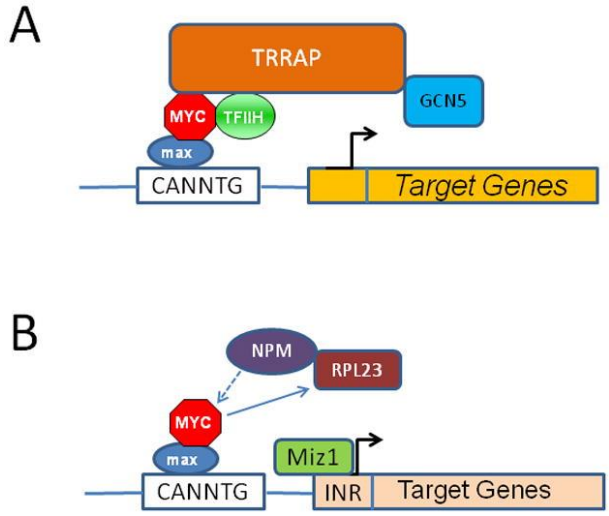


Figure 10. MYC-Max-Mediated Transcription and Gene Repression. (A) The MYC-Max heterodimer is shown to interact with key cofactors, such as transcription factor 2H (TFIIH), that triggers transcriptional elongation, or transformation/transcription domain-associated protein (TRRAP) that recruits the GCN5, which acetylates histones, permitting transcription of target genes. (B) MYC-Max also mediates gene repression. Miz-1 is shown tethered to the initiator (INR) element to regulate transcription of target genes, which could be silenced by MYC displacement of nucleophosmin (NPM), a Miz-1 cofactor, or MYC induction of the ribosomal protein RPL23, which retains NPM in the nucleolus, keeping it away from Miz-1. (Modified from Dang, Cell, 2012)

MYC act as a central hub of the cell, integrating signals from numerous pathways to control gene expression programs and regulate many biological functions, including cell growth, proliferation, apoptosis, differentiation, and transformation (Figure 11) [177]. MYC levels in cells are normally highly controlled at multiple levels, from MYC gene expression to protein stability, but become deregulated in many human cancers. Different cancer genome sequencing efforts affirm that MYC is one of the most frequently amplified genes in cancer [178]. MYC is deregulated in cancer in many ways [179]. For example, gene amplification, often used as a clinical marker of MYC aberration, often occurs in different types of cancer. Chromosomal translocation of MYC is found frequently in hematopoietic malignancies. Although generally considered an under-mutated gene, MYC is recurrently mutated in Burkitt's lymphoma [180]. Many additional mechanisms of MYC deregulation occur in cancer, including activation of upstream pathways, such as Wnt and Notch signaling, as well as increased mRNA and protein stabilization.

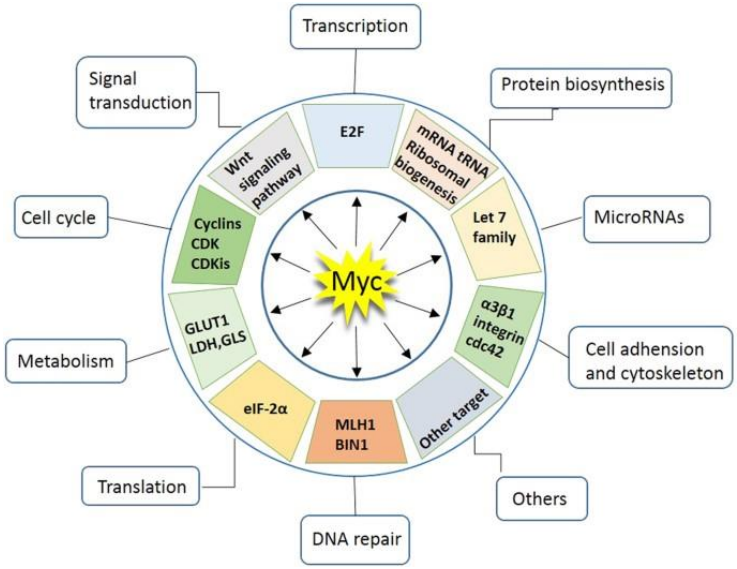


Figure 11. Signal transduction pathways controlled by MYC. (Modified from Chen et al., Signal Transduction and Targeted Therapy, 2018)

MYC is one of the most potent oncogenes as measured by many in vitro and in vivo assays for cell transformation phenotypes [181]. Although it is one of the most commonly activated oncogenes implicated in the pathogenesis of human cancers, MYC overexpression alone is surprisingly incapable of inducing cellular proliferation or neoplastic transformation of most normal human cells. Indeed, MYC-induced neoplastic transformation was detected only in specific cell lines, that were presumed to have acquired other genetic events that rendered them permissive [182]. Instead, MYC overexpression in normal cells can have no effects or can be highly destructive, culminating in proliferative arrest, senescence, and/or apoptosis. In the same way, MYC overexpression has been observed to enforce DNA replication and entry into S phase [183], as part of the replication complex [184], but MYC alone cannot cause mitotic cellular division [183]. Additionally, MYC overexpression can enforce replication in a manner that results in DNA breaks [185]. MYC overexpression can cause normal cells to undergo proliferative arrest under some circumstances [186] and cellular senescence in other cases [187]. Thus, MYC deregulation alone cannot force complete transit through the cell cycle division.

MYC overexpression consequences in a normal cell are also dependent on epigenetic and genetic contexts. For instance, in embryonic liver MYC overexpression induces cellular proliferation, whereas in adult liver it promotes cellular growth without mitotic division that is associated with polyploidy [188]. Circumstances that promote hepatocytes to proliferate, such as a treatment with liver toxin or a partial hepatectomy, can enable MYC to induce cellular proliferation in adult hepatocytes more readily [188]. Similarly, the loss of the tumor suppressor p53 cooperates with

MYC to induce cellular proliferation and tumorigenesis in adult hepatocytes. Thus, cellular context and specific genetic defects can enable MYC to induce proliferation and tumorigenesis. Moreover, also gene dosage of MYC strongly influences the consequences of its activation. Highly robust activation of MYC is more commonly associated with DNA damage and apoptosis; conversely, less robust MYC activation appears to be associated with proliferative arrest and cellular senescence [189].

3.4.2 MYC and neurodegeneration

Many studies have been conducted with the aim of unravelling the molecular links between cancer and neurodegeneration. MYC and its molecular partners have been implicated in the onset of neurodegenerative disorders. For instance, Lee and collaborators [190] generated an inducible mice model overexpressing human MYC under the control of the CaMKII promoter, that drives high transgene expression in forebrain neurons. Their findings showed that MYC expression in transgenic mice forebrain induced neuronal-specific cell cycle re-entry, neurodegeneration, and significant cognitive deficits. Moreover, the neurotoxic agents camptothecin and amyloid-β peptide in a differentiated SH-SY5Y neuronal cell culture model induce MYC overexpression followed by neuronal cell death [191].

Furthermore, other studies demonstrated the implications of MYC molecular partners in neurodegenerative diseases. Indeed, DNA-binding proteins associated to MYC seem to have big relevance. An example is DJ-1, an oncogene that accelerates cellular transformation by MYC. Cancer cells show elevated expression level of DJ-1, thus increasing Erk activity that culminates in the stimulation of cell growth [192]. Conversely, in animal models of and patients with PD, highly oxidized DJ-1 is accumulated, thus reducing Erk activity and thereby stimulating the apoptosis signaling pathway [193].

Concerning ALS, the link with MYC lies in an RNA binding protein, HuR. There are evidences that HuR plays a role in regulating ALS-associated RNA binding proteins and raise the possibility that HuR may contribute to motor neuron disease pathology by its unchecked positive regulation of ALS-associated TDP-43 and FUS/TLS [194]. Importantly, HuR is also involved in the modulation of MYC mRNAs, by directly binding and repressing it through recruitment of let-7 miRNA to an adjacent site on the MYC 3′UTR [195].

3.4.3 MYC-induced long non coding RNA (MINCR)

Recent studies have shown that altered expression of lncRNAs is linked to several disease conditions, particularly cancer [196]. Interestingly, transcriptome profiling of tumour samples has shown that many lncRNAs can be differentially regulated by MYC in different types of cancer and many of them can influence cancer cell viability

and proliferation. At the same time, lncRNAs are able to control the expression of MYC itself acting both at transcriptional and post-transcriptional levels [197]. About this point, Doose and collaborators [198] investigated the extent of changes that MYC can induce in lncRNA expression. The intersection of data from two different cell line model carrying a MYC-inducible construct and sequencing data from Burkitt Lymphoma patients' samples led to the identification of the MYC-induced long non-coding RNA (MINCR) (Figure 12).

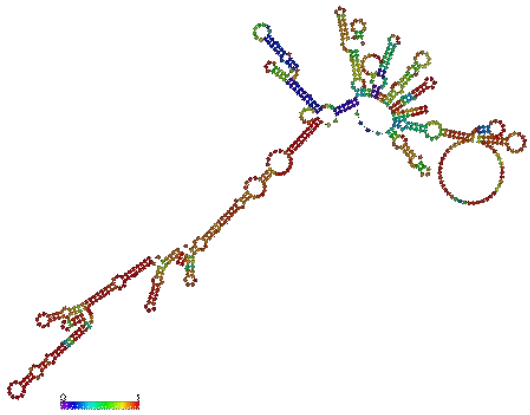


Figure 12. MINCR predicted secondary structure (RNAfold Web Server). Minimum free energy structure, colors correspond to base-pair probabilities

The MINCR gene is and intergenic lncRNA located between two coding genes GLI4 and ZNF696 on chromosome 8q24.3. At least six different isoforms transcribed from the MINCR gene locus are annotated on ENCODE, the longest isoform composed of three exons and all others containing two exons. MINCR showed a high correlation with MYC expression levels in MYC-positive lymphoma samples since MYC binds the transcription start site (TSS) region of MINCR promoting its transcription. Accordingly, they identified a significant correlation between MINCR and MYC expression in RNA-seq data from pancreatic ductal adenocarcinomas. Analysis of the transcriptional changes observed after MINCR knockdown in BL-2 and P493-6 cells suggested that the lncRNA MINCR is able to control the expression level of a set of genes involved in cell cycle initiation and progression. In particular, genes playing a role in initiation of genome replication and the assembly of the mitotic spindle are found strongly reduced after MINCR RNAi. MINCR, therefore, seems to be involved in controlling a subset of MYC target genes. Beside Burkitt lymphoma, MINCR is involved in other types of cancer, such as gallbladder cancer [199], hepatocellular carcinoma [200], non small-cell lung cancer [201], oral squamous cell carcinoma [202], nasopharyngeal carcinoma [203] and glioma [204]. In all these types of cancer MINCR is overexpressed and correlates with poor prognosis. Its mechanisms of

action is still unknown, even if recently has been suggested that it can act as competing endogenous RNA (ceRNA) binding to and modulating specific miRNAs [201] [203] [204].

Moreover, MINCR could be a key factor not only in cancer, but also in neurodegenerative diseases, considering its down-regulation sALS patients [86]. Rs7388117 MINCR variant of uncertain clinical significance, has been reported in temporal cortex and cerebellum of Alzheimer's disease (AD) patients [205]. This finding shed light on a potential pathogenetic role of MINCR in neurodegenerative diseases.

4. AIMS

Despite our knowledge has increased in the last years, ALS still remains a pathology of uncertain etiology. To make the scenario even more complicated, ALS is a multifactorial disease generated by the interplay between many different processes and molecules. One of the emerging involved processes is RNA metabolism, both involving coding and non coding transcripts. Dysregulation in RNA-binding proteins, in gene expression and in transcription factor activity have emerged. In this context, we performed a whole transcriptome analysis in PBMCs of sALS patients compared to healthy controls that revealed two main concepts: a deregulation in genes involved in transcription and a significative alteration of lncRNA expression [86]. In the light of this considerations, we found interesting the deregulation of MYCBP and MINCR RNA levels in sALS, both related to the transcription factor MYC.

Therefore, starting from these data, the aims of this work were:

1. To identify the most involved transcription network in order to understand the causes of its alteration and the consequences on gene expression and RNA metabolism in sALS. To achieve this result, we analyzed RNA-seq data and confirmed the alteration of MYC and related genes, MYCBP and MINCR, through qPCR validation. Moreover, we showed that almost the 90% of differentially expressed genes in transcriptomic data from sALS patients are MYC targets, highlighting a central role for MYC in RNA deregulation. Starting from these data, we decided to investigate the MYC transcription factor in sALS.

2. To explore lncRNA involvement in the pathogenesis of sALS. For this aim we focused our attention on the MYC induced lncRNA MINCR, investigating its features, targets and mechanisms of action. To achieve this result, we firstly investigated the relationship between MINCR and MYC. Secondly, we characterized the effect on gene expression of overexpression and knockdown of MINCR in SH-SY5Y neuroblastoma cells. Finally, we studied MINCR action in ALS knocking-out MINCR through CRISPR-Cas9 system and finding its protein interactor through RNA pull down in differentiated SH-SY5Y cells.

5. MATHERIALS AND METHODS

5.1 Patients and control subjects

30 SALS patients and 30 age- and sex-matched healthy controls (CTR) were recruited after obtaining written informed consent (Table 1). A subset of subjects (10 ALS and 3 CTR) was deep-sequenced while all samples were included for Real Time PCR experiments. ALS patients underwent clinical and neurologic examination at IRCCS Mondino Foundation (Pavia, Italy). All patients were diagnosed with ALS as defined by El Escorial criteria. All SALS patients were analyzed to exclude any causative mutations in SOD1, TARDBP, FUS, C9orf72, ANG and VCP genes. The control subjects were recruited at the Transfusional Service and Centre of Transplantation Immunology, Foundation San Matteo, IRCCS (Pavia, Italy). The study protocol to obtain PBMCs from patients and controls was approved by the Ethical Committee of the IRCCS Mondino Foundation (Pavia, Italy). Before being enrolled, the subjects participating in the study signed an informed consent form (Protocol n°375/04 – version 07/01/2004). Spinal cord tissue was obtained from the Human Brain and Spinal Fluid Resource Center (VA West Los Angeles Healthcare center, Los Angeles, CA 90073), which is sponsored by NINDS/NIMH, National Multiple Sclerosis Society, and Department of Veteran Affairs. All experiments were performed in accordance with relevant guidelines and regulations.

	CTRs	SALS
	(n = 30)	(n = 30)
Age (M ± SD)	49 ± 10,3	66,6 ± 10,1
Sex		
Males n (%)	48%	45%
Females n (%)	52%	55%
Onset		Spinal (100%)
ALSFRS		41,15 [39,42–42,88]

Table 1. Baseline characteristics of subjects recruited for this study. Age values are reported as average ± standard deviation. The percentage of male and female subjects and the site of onset are also indicated. ALSFRS score is indicated as average and 95% confidence interval.

5.2 PBMCs extraction from blood samples

PBMCs were immediately isolated from peripheral venous blood using Histopaque®-1077 (Sigma-Aldrich, Italy) following manufacturer's instructions. PBMCs (composed of ~80% lymphocytes and ~20% of monocytes) were layered on Histopaque®-1077, centrifuged at 1800 rpm for 30 minutes, then washed with 1X PBS and centrifuged again at 1600 rpm for 10 minutes. Cells viability was assessed by trypan blue exclusion test.

5.3 RNA extraction

Total RNA from PBMCs, spinal cord and SH-SY5Y cell line was isolated by Trizol® reagent (Life Science Technologies) following the manufacturer's specifications. RNAs were quantified and examined using Nanodrop ND-100 Spectrophotometer (Nanodrop Technologies) and 2100 Bioanalyzer (Agilent RNA 6000 Nano Kit).

Cytoplasmic and nuclear RNA fractions were extracted using the Cytoplasmic & Nuclear RNA Purification Kit (Norgen Biotek Corp.) according to the manufacturer's instructions.

5.4 cDNA synthesis, qPCR and ddPCR

Total cDNAs were prepared from 1 μg of total RNA using iScript™ cDNA Synthesis Kit (Bio-Rad). qPCR was performed according to manufacturer's instructions with iQ™ SYBR® Green Supermix (Bio-Rad). Primers were designed with Primer3Plus software (Table 2) [206]. Genes were quantified in triplicates, GAPDH was used as housekeeping gene. Gene expression was calculated using the 2-ΔΔCt method.

Droplet Digital PCR (ddPCR) reaction mixture contained 2x EVAGreen Supermix (no dUTP) (Bio-Rad), 100 nM of each forward and reverse primers and 5 ng of cDNA. Droplet emulsion was generated using QX 200 droplet generator (Bio-Rad) and analyzed with QX 200 droplet reader (Bio-Rad).

Gene	Sequence
MAX Forward	5'- AAGCTGCTTGAGGGCTGATA-3'
MAX Reverse	5'- TTCAAAAATGGAACCCCATC-3'
MINCR Forward	5'- TGGAATTTCAAGCCCAACTC-3'
MINCR Reverse	5'- CTTGACAGCTGCAGGTCTTG-3'
MYCBP Forward	5'- TTGTACGGGTTCCCATGAAT-3'
MYCBP Reverse	5'- AACAGCACAGAAAGGCCAGT-3'
MYC Forward	5'- AGCAATCTGGACCCATTCTG-3'
MYC Reverse	5'- GGAGTGCTTGGGACACAAAT-3'

Table 2. Oligonucleotide sequences used for qPCR.

5.5 Protein extraction and quantification

Cell pellet was lysed in 70 µl of cold Radio-Immunoprecipitation Assay (RIPA) buffer (150 mM sodium chloride, 1.0% NP-40 or Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0), containing a mixture of phosphatase and protease inhibitors (Sigma-Aldrich), incubated for 20 minutes in ice and centrifuged at 16,000 g for 20 min at 4°C. The supernatant was transferred to a fresh tube and stored in aliquots at -80°C, representing the total protein extract, together with the remaining pellet of insoluble proteins.

The subcellular fractionation was performed according to the method of Schreiber and colleagues [207], with some modifications. After cells have been washed with ice-cold 1X PBS, the cellular pellet was resuspended in ice-cold hypotonic lysis buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 1% of protease and phosphatase inhibitor cocktail). Cells were allowed to swell on ice for 25 min, after which 25 µL of 10% Nonidet NP-40 (Fluka, St. Gallen, Switzerland) was added. Samples were vortexed and centrifuged at the maximum speed. The supernatant, containing the cytoplasm proteins, was collected and stored at –80 °C until the use. The nuclear pellets were resuspended in ice-cold hypertonic nuclear extraction buffer (20 mM HEPES, pH 7.9,

0.4 M NaCl, 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1% of protease and phosphatase inhibitor cocktail), and incubated on ice for 20 min with agitation. The nuclear extracts were then centrifuged at the maximum speed for 5 min at 4 °C and the supernatant containing the nuclear proteins was collected and frozen at –80 °C.

Protein concentration was determined using bicinchoninic acid (BCA) method (Sigma-Aldrich) and BSA (bovine serum albumin) as standard. A solution of copper (Cu+2) and BCA was prepared 1:50 and 20 µl of this solution were mixed with 2,5 µl of an intermediate dilution 1:10 of each sample. The principle of this method is that proteins can reduce Cu+2 to Cu+1 in an alkaline solution (the biuret reaction) resulting in a purple color formation which is directly proportional to the protein component present. The samples were incubated at 37°C, temperature requested for the formation of peptide bonds involved in reaction complex development. Protein quantification was determined using Nanodrop ND-100 Spectrophotometer (Nanodrop Technologies).

5.6 Western Blot WB

WB analysis was performed by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) on PBMCs of sALS patients and healthy control subjects. Samples containing 30 µg of proteins were boiled at 80°C in Laemmli sample buffer (0.6 g/100 ml Tris, 2 g/100 ml SDS, 10% glycerol, 1% β-mercaptoethanol, pH 6.8) for 10 min and then loaded on 12.5% SDS-PAGE gel (Bio-Rad). After electrophoresis, samples were transferred to a PVDF (Polyvinylidene difluoride) membrane (Trans-blot, Bio-Rad) using a liquid transfer apparatus (Bio-Rad). PVDF membranes were treated with a blocking solution containing 5% Bovine Serum Albumin (Sigma-Aldrich) in TBS-T buffer (10 mM Tris-HCl, 100 mM NaCl, 0.1% Tween, pH 7.5) for 1h to block unspecific protein binding sites and then were incubated overnight with the primary antibodies prepared in blocking solution at 4°C in continuous shaking. The list of all antibodies used in this project is shown in Table 3. Immunoreactivity was detected using the donkey anti-rabbit or anti-mouse (GE Healthcare) secondary peroxidase-conjugated antibodies (Table 3). The immunoreactive bands were then visualized using the enhanced chemiluminescence detection kit (ECL Select, GE Healthcare).

Antibody	Species	Concentration	Supplier
Anti-c-MYC	Rabbit	1:5000 (WB) 1:200 (IF) 2 µg (RIP)	Abcam
Anti-c-MYC	Mouse	4 µg (ChIP)	Santa Cruz Biotech
Anti-MYCBP	Mouse	1:200 (WB)	Santa Cruz Biotech
Anti-MAX	Mouse	1:1000 (WB) 2 µg (IP) 1:100 (IF)	Santa Cruz Biotech
Anti-GAPDH	Rabbit	1:10000 (WB)	Genetex
Anti-Beta-Tubulin III	Rabbit	1:1000 (IF)	Abcam
Anti-Nestin	Mouse	1:200 (IF)	Abcam

Table 3. Antibodies used to perform western blotting (WB), immunoprecipitation assay (IP), RNA immunoprecipitation (RIP), immunofluorescence assay (IF) and chromatin immunoprecipitation assay (ChIP)

5.7 Immunoprecipitation IP

After a pre-clearing phase of 2 h at 4 °C, IP was carried out in PBMCs of sALS patients and healthy subjects at 4 °C using 2 µg of mouse monoclonal primary antibody anti-MAX (Santa Cruz Biotechnology) per 500 µg of proteins diluted in RIPA buffer (1% of protease and phosphatase inhibitor cocktail) on overnight incubation, followed by incubation with 20 µl of Dynabeads™ Protein G (Invitrogen). The samples were finally subjected to Western blotting for: Anti-c-MYC (Abcam) and Anti-MAX (Santa Cruz Biotechnology). IP- and IgG were used as controls.

5.8 Immunofluorescence IF

PBMCs were harvested, washed with 1X PBS and suspended in RPMI-1640 medium. About 1 x 10^5 cells were placed on a poly-L-Lysine slide (ThermoFisher Scientific) and incubated at 37°C for 30 min to allow cell attachment to the slide. Cells were rinsed with 1X PBS and then fixed using a solution of 4% paraformaldehyde in 1X PBS.

About 2x10^4 wild-type and MINCR-knocked-out SH-SY5Y were grown and differentiated on 0.5% gelatin coated glass coverslip, rinsed with 1X PBS and fixed with 4% paraformaldehyde in 1X PBS.

Fixed cells were washed with 1X PBS and treated with a blocking solution (5% normal goat serum in 0.1% Tween-PBS) for 1 h with the aim of blocking unspecific protein binding sites, slides were then incubated overnight at 4°C with primary antibodies: Rabbit monoclonal anti-c-MYC (1:200) (Abcam), Mouse monoclonal anti-MAX (1:100) (Santa Cruz Biotechnology), Rabbit monoclonal anti-TUBB3 (1:1000) (Abcam) and Mouse monoclonal anti-Nestin (1:200) (Abcam). Cells were washed with 1X PBS and incubated at room temperature for 1 h with secondary antibodies: CFTM 488A goat anti-rabbit (1:700 dilution) (Invitrogen) and CFTM 594 goat anti-mouse (1:700 dilution) (Invitrogen). Both primary and secondary antibodies were prepared in blocking buffer. Finally, slides were washed with 1X PBS, mounted with Prolong® Gold antifade reagent with DAPI (Invitrogen), dried, nail-polished and analyzed by confocal microscopy (Leica DM IRBE, Leica Microsystems Srl, Wetzlar, Germany).

5.9 Chromatin immunoprecipitation ChIP

ChIP assay was performed starting from 20x10^6 PBMCs of sALS patients and control subjects with ChIP-IT® PBMC kit (Active Motif) according to the manufacturer's instruction. Briefly, intact cells were fixed with formaldehyde, which cross-links and preserves protein/DNA interactions. DNA was then sheared into small fragments using sonication and incubated with 4 µg anti-c-MYC antibody (Santa Cruz Biotechnology). The antibody-bound protein/DNA complexes were immunoprecipitated through the use of Protein G agarose beads and washed via gravity filtration. Following immunoprecipitation, cross-links were reversed, the proteins were removed by Proteinase K and the DNA was recovered and purified. ChIP enriched DNA was used for qPCR, that was performed as previously described using samples incubated with IgG antibody as negative controls. Primers for the promoter region of MINCR were designed with UCSC Genome Browser and Primer3Plus (Table 4).

Gene	Sequence
MINCR-promoter Forward	5'-ATAACCCTGTGAGTGCATTCCT-3'
MINCR-promoter Reverse	5'-GCTAAGAAAATCGACCTTGGAA-3'

Table 4. Oligonucleotide sequences used for qPCR.

5.10 Cell culture and differentiation

The human neuroblastoma cell line SH-SY5Y (ATCC) was grown in DMEM (Invitrogen) supplemented with 15% fetal bovine serum (Carlo Erba), 1% L-Glutammine (Carlo Erba) and 1% penicillin/streptoMYCin (Carlo Erba), at 37 °C in a 5% CO_2 atmosphere.

Cell differentiation was performed following the protocol proposed by Forster et al. [208]. Briefly, cells were cultivated in DMEM (Invitrogen) with 1% P/S further supplemented with 10 μM all-trans retinoic acid (Sigma Aldrich) for 3 days. Then, the medium was changed with Neurobasal-A medium minus phenol red (Invitrogen) with 1% l-glutamine, 1% P/S, 1% N-2 supplement 100X (Invitrogen) and human brain derived neurotrophic factor BDNF (Invitrogen) at the concentration of 50 ng/mL for 3 more days, for a total of 6 days of treatment.

5.11 Cell stable transfection and silencing

For MINCR stable transfection (SH-MINCR+), a pCMV6-AC-GFP construct with neomycin resistance (OriGene) was used (Figure 13). The plasmids have been transformed in E. Coli, we have picked individual colonies and checked them for successful ligations by growing cultures, DNA purification, agarose gel run and sequence.

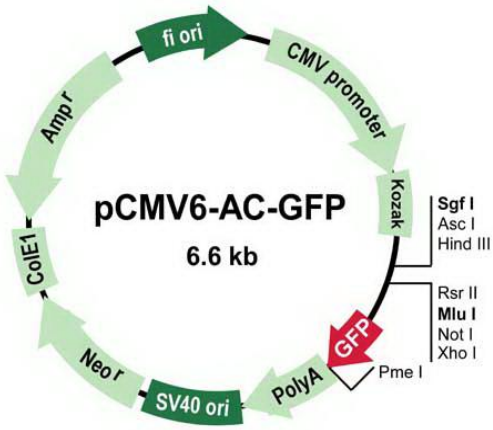


Figure 13. pCMV6-AC-GFP construct used for MINCR stable transfection.

For transfection cells were seeded in 24-well plates at a density of 6×10^4 cells/well. After 24 h, cells were transfected with 500 ng of plasmid (pCMV6-AC-GFP and empty vector as negative control) and FugeneHD transfection reagent (Roche). After 48 h incubation, the culture medium was changed, and cells were cultured with selective medium containing 600 µg/ml gentamicin-disulfate G418 (Roth). After 3 weeks of G418 selection, single clones were isolated and cultured in 48-well plate. The transfection efficiency was observed through qPCR and calculating the % of GFP positive cells observed by fluorescence microscopy through ImageJ "Cell counter" plug-in.

For MINCR silencing (SH-siMINCR), SH-SY5Y cells were seeded in a 6 well-plate at a density of $4x10^5$ cells/well the day before transfection. The cells were transiently transfected with siRNA-MINCR designed on the longest isoform (ThermoFisher) and siNEG, as negative control, using Lipofectamine® RNAiMAX Transfection Reagent (Invitrogen) according to the manufacturer's instructions. Cells were cultured in DMEM Low Glucose without antibiotics and harvested after 72 hours of transfection. The silencing efficiency was observed through qPCR.

5.12 RNA immunoprecipitation RIP

RIP was carried out with $6x10^6$ PBMCs from sALS patients and matched controls. Cells were washed and harvested in ice-cold PBS and lysed in hypotonic gentle lysis buffer (10 mM Tris-HCl pH 7.5, 10 mM NaCl, 2mM EDTA, 0.5% Triton X-100, RNasin and protease inhibitor). The lysate was incubated on ice for 10 minutes and added with NaCl to 150 mM. Then, the cells were centrifuged at 14000 rpm for 15 minutes. The cell lysate was immunoprecipitated with antibody Anti-MYC (Abcam) and anti-IgG overnight at 4°C. Samples were incubated with Dynabeads™ Protein G (Invitrogen) and the RIP fractions were washed 5 times with wash buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl and 0.05% Triton X-100). The immunoprecipitated

RNA was isolated with Trizol protocol and analyzed by RNA-seq and ddPCR. The results were expressed as the relative fold enrichment of the target precipitation compared with the IgG control.

5.13 Library preparation for RNA-seq and bioinformatic data analysis

Purified RNA from SH-MOCK, SH-MINCR+ and SH-siMINCR were prepared with the SENSE Total RNA-Seq Library Prep Kit (Lexogen) using 500 ng total RNA and sequenced in paired-end by Illumina NextSeq 550 sequencer. The quality of sequencing libraries were assessed by 2100 Bioanalyzer with DNA1000 assay (Agilent) and quantified with Qubit™ dsDNA HS Assay Kit (Invitrogen). FastQ files were generated via Illumina bcl2fastq2 starting from raw sequencing reads produced by Illumina NextSeq sequencer (Version 2.17.1.14- http://support.illumina.com/downloads/bcl-2fastq-conversion-software-v217.html). Gene and transcript intensities were computed using STAR/RSEM software using Gencode Release 27 (GRCh38) as a reference, using the "stranded" option. Differential expression analysis for mRNA was performed using R package DESeq.2. Coding and non coding genes were considered differentially expressed and retained for further analysis with $|\log2(\text{SH-MINCR+/SH-MOCK})| \geq 1$ and a False Discovery Rate ≤ 0.1.

5.14 Pathway analysis and Gene Ontology

We performed KEGG pathway analysis (Kyoto Encyclopedia of Genes and Genomes http://www.genome.ad.jp/KEGG), WikiPathways analysis and Gene Ontologies of differentially expressed coding genes via enrichR web tool [209]. The R software was used to generate Dotplot graphs (with the ggplot2 library) and GOChord graphs (with the GOplot library). All other representations of functional enrichment were generated using the Cytoscape software [210] and the following plugins: NDEx [211], ClueGO [212], DisGeNET [213] and BinGO [214].

5.15 RNA Pull down

RNA pull-down was performed as described in Marín-Béjar et al. [215]. Firstly, the MINCR sequence from pcDNA™3.1 plasmid (ThermoFisher Scientific) was subcloned into the PT3T7 plasmid under the control of the T7 promoter, for MINCR in vitro transcription, and of the T3 promoter, for MINCR antisense in vitro transcription used as negative control (Figure 14). pcDNA™ 3.1 with MINCR sequence was double digested with EcoRI and NotI restriction enzymes, MINCR band was isolated and purified from 1% agarose gel and then ligated to PT3T7 plasmid, already double digested with EcoRI and NotI too.

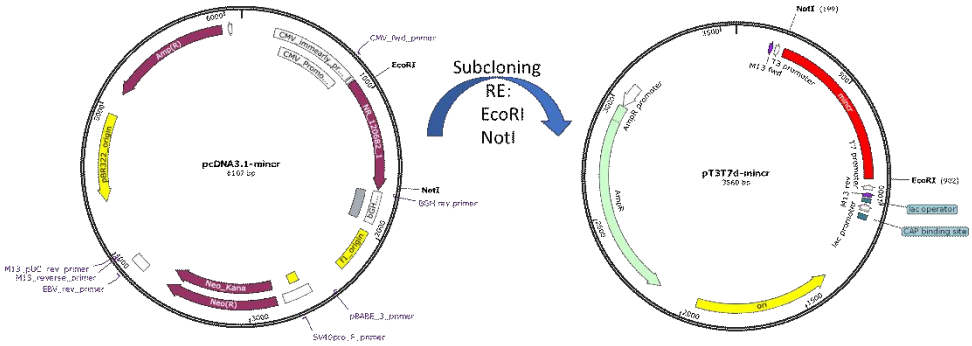


Figure 14. Subcloning of MINCR sequence from the pcDNA™ 3.1 vector to PT3T7 vector. Restriction Enzymes (RE).

To check the correct insertion of MINCR sequence in PT3T7 plasmid, we sequenced MINCR through Sanger sequencing and we performed PCR after double digestion with different pairs of restriction enzymes, that generate fragments of different sizes, then we visualized and checked the fragment size on 1% agarose gel.

PT3T7 plasmid with MINCR sequence was expanded in E. Coli competent cells and used for in vitro transcription and RNA biotinylation:

- for MINCR transcription the plasmid was cut with NotI and transcribed under the control of T7 promoter
- for negative control, MINCR antisense was transcribed under the control of T3 promoter after cut with EcoRI.

Size of MINCR and antisense transcript were confirmed by visualization on 1% agarose gel.

Since MINCR is a predominantly nuclear RNA, we performed RNA pull down on nuclear fraction of differentiated SH-SY5Y cells. Nuclear extract was performed starting from 2 x 10^7 cells. After trypsinization, cells were resuspend in 4 mL of cold 1X PBS + 4 mL of Nuclear Isolation Buffer (1.28 M sucrose; 40 mM Tris–HCl pH 7.5; 20 mM MgCl2; 4 % Triton X-100) + 4 mL of H_2O and left on ice for 15 min. Then, the cells were centrifuge for 15 min at 2500 rpm at 4 °C to pellet nuclei. Nuclear pellet was resuspended in 2 mL of Buffer A (150 mM KCl, 25 mM Tris pH 7.4, 5 mM EDTA, 0.5 mM DTT, 0.5 % NP40, 9 µg/ml leupeptin, 9 µg/ml pepstatin, 10 µg/ml chymostatin, 3 µg/ml aprotinin, 1 mM PMSF, 100 U/ml SUPERASin) and transferred to dounce homogenizer to shear mechanically using 15-20 strokes. Then nuclear membranes and debris were pelleted by centrifugation at 13000 × g for 10 min. the supernatant, containing the nuclear extract, was used for RNA pull down.

After a first step of preclearing with 65 µL of Dynabeads™ MyOne™ Streptavidin C1 (Invitrogen), nuclear extract, supplemented with yeast tRNA (0.1 µg/µl), was

incubated with 10 µg of biotinylated RNA of MINCR and MINCR antisense for 1 h and 30 min at 4 °C in a rotary shaker. To isolate biotinylated RNA bound to protein, 30 µL of Dynabeads™ MyOne™ Streptavidin C1 (Invitrogen) washed and resuspended in 100 µL of Buffer A per condition were added and incubated for 1 h at 4 °C on a rotary shaker. Beads were washed 5 times with Buffer B (300 mM KCl, 25 mM Tris pH 7.4, 5 mM EDTA, 0.5 mM DTT, 0.5 % NP40, 9 µg/ml leupeptin, 9 µg/ml pepstatin, 10 µg/ml chymostatin, 3 µg/ml aprotinin, 1 mM PMSF, 100 U/ml SUPERASin) and then resuspended in 36 µL of LB 4X. After boiling the sample at 95 °C for 10 min and removing the beads, interacting proteins were loaded in a NuPAGE Novex 4–12% bis-Tris gel (Invitrogen) and stained with SilverQuest Silver Staining Kit (ThermoFisher Scientific). One differential band was seen (Figure 15) and sent for mass spectrometry to Taplin Mass Spectrometry Facility (Harvard Medical School; USA).

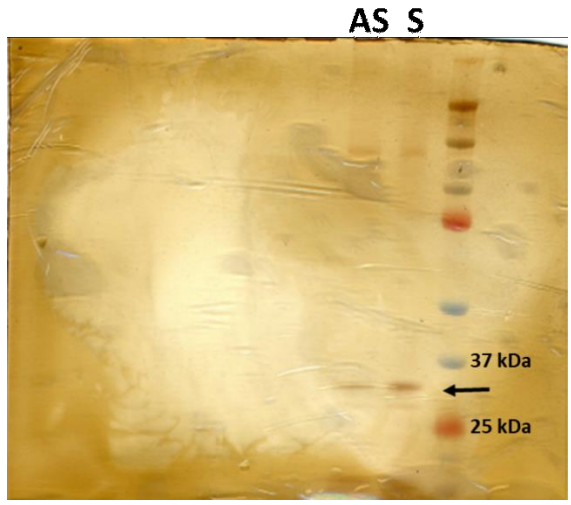


Figure 15. RNA pull down gel of MINCR sense (S) and antisense (AS) transcripts. The arrow shows the bands sent for mass spectrometry.

5.16 CRISPR-Cas9 editing

Two single-guide RNAs (sgRNAs) were designed to delete the entire MINCR gene using a tool from the Zhang Lab (http://crispr.mit.edu/) (Table 5). Oligonucleotides to clone the guide RNA were then annealed and cloned into pX330 vector (Figure 16) containing Cas9 [216]. The correct insertion and sequence of the annealed sgRNAs were confirmed by PCR on single colony and with Sanger sequencing. Then pX330-sgRNA1 and pX330-sgRNA2 plasmids were co-transfected with GFP expressing plasmid (pmax-GFP) into SH-SY5Y cells.

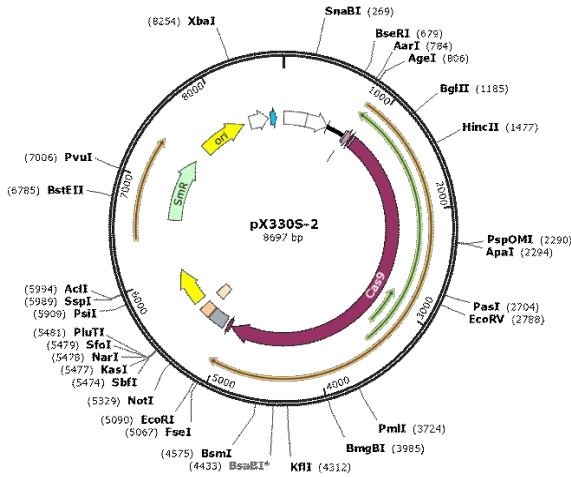


Figure 16. pX330s-2 vector containing Cas9 and sgRNA cloning site

GFP positive cells were sorted in 96 well plate by BD FACSAria IIu cytometer. After single cells reached confluency, genomic DNA was extracted using QuickExtract reagent (Epicentre) and PCR was performed using a pair of primers flanking the depleted region and positive clones were identified by PCR product length. Furthermore, RNA was extracted to perform qPCR to validate deletion of aimed region (Table 5).

Oligonucleotides	**Sequence**
sgRNA 1.1	5’ – CACCGGTTCGGAAGACACGCGACCA – 3’
sgRNA 1.2	5’ – AAACTGGTCGCGTGTCTTCCGAAC – 3’
sgRNA 2.1	5’ – CACCGTTGGCTAAGAAAATCGACCT – 3’
sgRNA 2.2	5’ – AAACAGGTCGATTTTCTTAGCCAAC – 3’
PCR F1	5’- CACTGGAGCTGCAGGGAGAGGT – 3’
PCR R1	5’ – TGACCCTCAGCTCCACCTCTGC – 3’
PCR F2	5’ – CAGTTAACTGTGAGGTTGGATGAGT – 3’
PCR R2	5’ – TGCTGAGTGTGTCTGTGGGGGT – 3’
qPCR F	5’ – AGCAATCTGGACCCATTCTG – 3’
qPCR R	5’ – GGAGTGCTTGGGACACAAAT – 3’

Table 5. Oligonucleotide sequences used for performing (sgRNA) and checking (PCR, qPCR) CRISPR-Cas9 genome editing

5.17 Apoptosis assay

Apoptosis assay on differentiated wild-type SH-SY5Y and differentiated SH-SY5Y knockout for MINR (SH-KO) was performed by Annexin V and 7-AAD staining using Apoptosis Detection Kit I (BD Biosciences) according to manufacturer’s instructions and detected by FACSCalibur flowcytometer.

6. RESULTS

6.1 MYC network alteration in sALS

6.1.1 MYC and its molecular partner RNAs are deregulated in PBMCs and spinal cord of sporadic ALS patients

Starting from RNA-seq data of 10 sALS patients published by our group [86], we performed Real Time PCR (qPCR) analysis in order to validate the results. qPCR results confirmed RNA-seq data, showing significant decrease of MINCR and MYCBP in PBMCs of sALS patients compared to healthy controls, while MYC level appears to be higher in patients compared to controls and MAX didn't show a significant variation (Figure 17, panel A). These alterations were confirmed also in spinal cord, as main involved tissue in ALS, corroborating the data and the use of PBMCs (Fig 17, panel B).

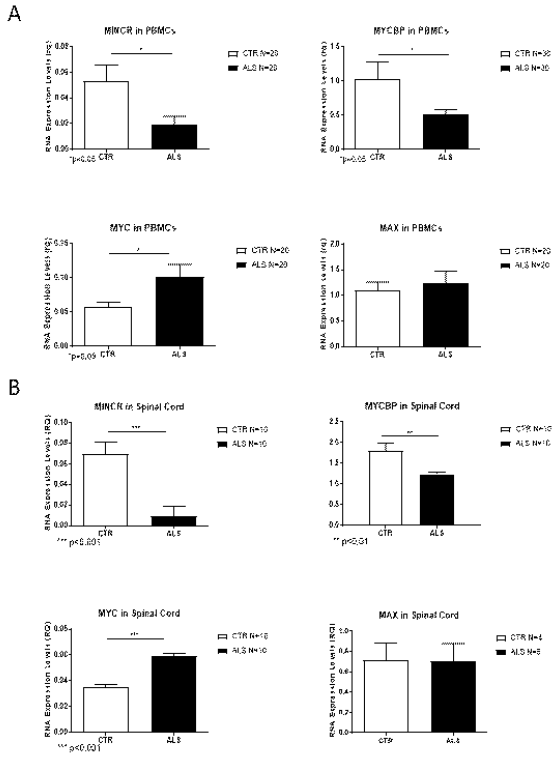
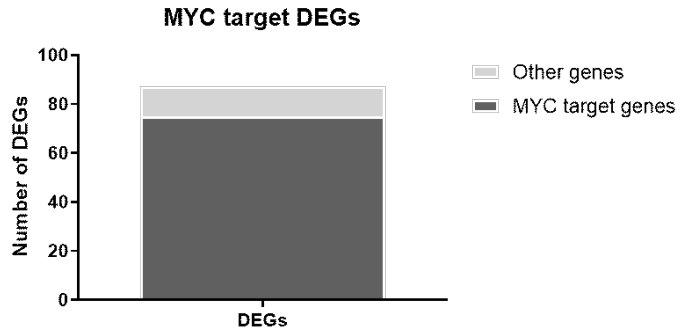


Figure 17. Expression analysis of MINCR, MYC and MYC's interactors MYCBP and MAX in PBMCs (A) and spinal cord (B) from sALS patients (ALS) and heathy subjects (CTR).

6.1.2 The major part of deregulated genes in sALS are MYC targets

Since MYC is a fundamental transcription factor, we wondered if its alteration could contribute to the gene expression deregulation found in PBMCs of sALS patients. Through the "ENCODE Transcription Factor Targets Dataset" [217], we were able to analyze differentially expressed coding genes (DEGs) to determine whether or not they were targets of MYC. As shown in Figure 18 and Table 6, 75 coding DEGs out of 87 (86.2%) resulted to be MYC targets. The list includes both down-regulated and up-regulated genes, and this is not surprising since, beside the well-known activity of MYC as transcriptional activator, it can act suppressing gene transcription too as heterodimer with Max and interacting with other co-factors (proteins or non coding RNAs). These data suggested a great alteration in MYC network and a potentially central role for MYC in RNA metabolism dysregulation of sALS.



	MYC target	Other genes	Total
DEGs down-regulated	28	3	31
DEGs up-regulated	48	8	56
DEGs total	75	12	87

Figure 18. Coding DEGs in PBMCs of sALS patients. 75 DEGs out of 87 are MYC targets based on the "ENCODE Transcription Factor Targets Dataset".

MYC target DEGs					
DEGs	Fold Change	DEGs	Fold Change	DEGs	Fold Change
OR2B11	2.35	**SCRN3**	1.25	**PTGER2**	-1.01
CCDC85C	2.28	**ACVR2A**	1.24	**TRIM13**	-1.05
TMEM236	2.23	**SLC1A4**	1.20	**ZC3H13**	-1.05
RGAG4	2.07	**RHOQ**	1.19	**TPCN1**	-1.06
PGM5P2	2.07	**EML4**	1.18	**SH2B3**	-1.10
PSIP1	2.01	**CDC42EP3**	1.17	**ANKS1B**	-1.15
RIC1	1.90	**FPR3**	1.16	**PA2G4**	-1.18
SMURF1	1.82	**POU2F2**	1.16	**PRH2**	-1.22
CCT6P3	1.80	**BABAM1**	1.15	**A2M**	-1.23
BRAT1	1.71	**UHRF1**	1.14	**VAMP1**	-1.23
LOC100507557	1.67	**ATP8B1**	1.14	**C11orf65**	-1.24
L3MBTL3	1.64	**MYO5B**	1.11	**USP35**	-1.33
E2F3	1.62	**EFCAB13**	1.10	**AHNAK**	-1.54
MAK	1.59	**CNP**	1.09	**TMEM132A**	-1.74
TCOF1	1.51	**TTC25**	1.08	**SPON1**	-1.96
C5orf56	1.47	**CHRNE**	1.06	**ENTPD7**	-2.03
SLC22A5	1.45	**TAF1C**	1.06	**PDSS1**	-2.04
F2RL2	1.42	**ESRP2**	1.06	**OPN3**	-2.13
NAIP	1.42	**FHOD1**	1.06	**TAF5L**	-2.21
SH3D19	1.41	**MPV17L**	1.05	**LOC400794**	-2.32
SLC26A1	1.40	**C16orf89**	1.02	**TTF2**	-2.52
TTC28	1.39	**UBN1**	1.01	**MYCBP**	-2.67
PDCD1	1.33	**VASN**	1.00	**ZMYM6**	-2.72
RAPH1	1.33	**ADCY9**	-1.00	**HDAC1**	-2.72
NFE2L2	1.27	**GPR65**	-1.01	**KIAA2013**	-3.09

Table 6. MYC target coding DEGs in PBMCs of sALS patients. Gene name and Fold Change are reported. Only genes with p-value<0.05 are shown.

6.1.3 Protein levels of MYC and its interactors are comparable between sALS patients and healthy controls

MYC, MYCBP and Max protein levels were evaluated by Western Blot in PBMCs with the aim of understanding if the deregulation in MYC target DEGs could depend on changes of protein levels. In control subjects and sALS patients MYC, MYCBP and Max levels were similar without statistically differences (Figure 19).

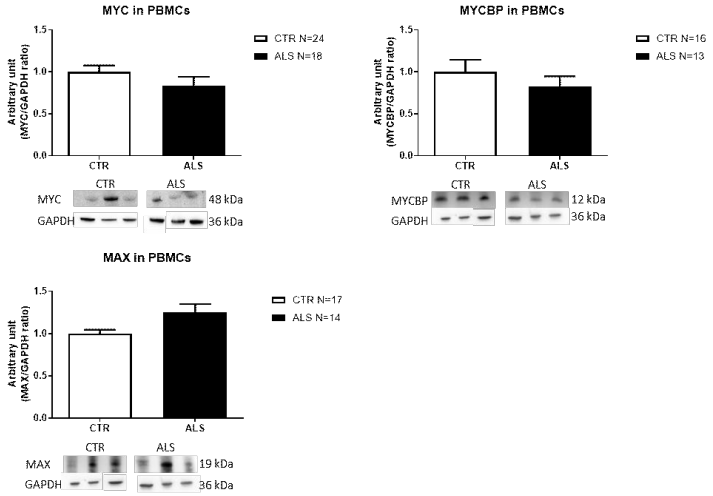


Figure 19. MYC, MYCBP and Max protein levels detected by Western Blot in PBMCs of sALS patients (ALS) and heathy controls (CTR).

6.1.4 MYC/Max complex dimerization is impaired in PBMCs of sALS patients

Since Western Blot analysis did not reveal any deregulation in protein level of MYC, MYCBP and Max, we assessed the transcriptional activity of MYC investigating the heterodimer formation with Max. Therefore, we performed Max immunoprecipitation (IP) and then we evaluated the levels of bound MYC. IP experiments showed that MYC binds Max more in control subjects than in sALS patients (Figure 20A), revealing that in ALS the MYC/Max heterodimer formation is impaired, probably causing a reduced activity of the complex. Moreover, immunofluorescence analysis (IF) of MYC and Max in PBMCs of sALS patients and healthy subjects confirmed the IP data showing the co-localization of MYC and Max mostly in control subjects (Figure 20B).

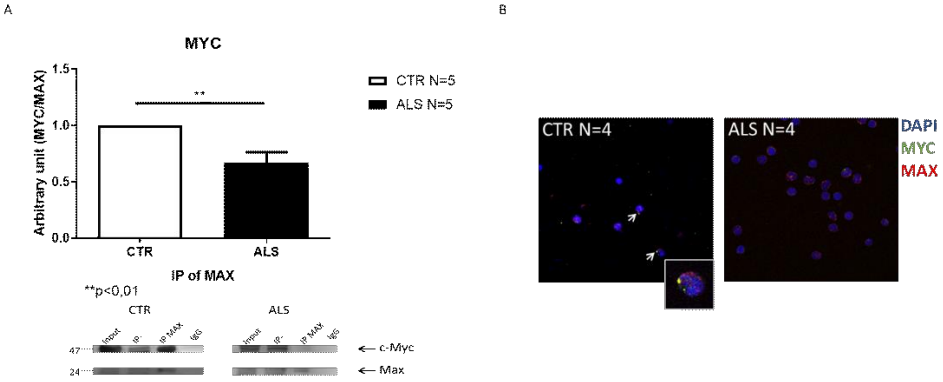


Figure 20: (A) IP of Max and MYC evaluation in PBMCs of sALS patients (ALS) and control subjects (CTR). (B) IF of MYC and Max in PBMCs of sALS patients (ALS) and control subjects (CTR). MYC: green, Max: red, co-localization: yellow.

6.1.5 MYC subcellular distribution is different in sALS patients

MYC is mainly localized in the nucleoplasm where it exerts its function and dimerizes with Max [218]. Mislocalization of MYC and other transcription factors have already been reported and this type of intracellular movement not only regulates protein localization, but also impacts on function [219] [220]. We investigated the subcellular localization of MYC in PBMCs of sALS patients through Western Blot analysis of cytoplasmic and nuclear fraction, showing that MYC distribution is altered: high MYC levels in cytoplasm and low MYC levels in nucleus (Figure 21). These data suggest that the MYC/Max heterodimer impairment in sALS could be not a problem of protein quantity, indeed MYC and Max protein levels are similar between sALS patients and controls, but could be a problem of protein mislocalization.

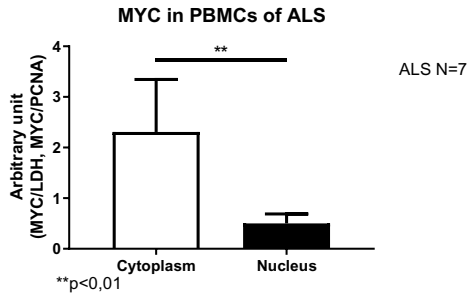


Figure 21. MYC subcellular localization in PBMCs of sALS patients. Western Blot analysis of cytoplasmic and nuclear fraction.

6.1.6 MYC binds the TSS region of MINCR less in sALS patients

Since our data showed that the MYC/Max heterodimer formation is impaired, our hypothesis is that MYC transcriptional activity network could likely be dysregulated leading to gene expression alterations (Figure 22).

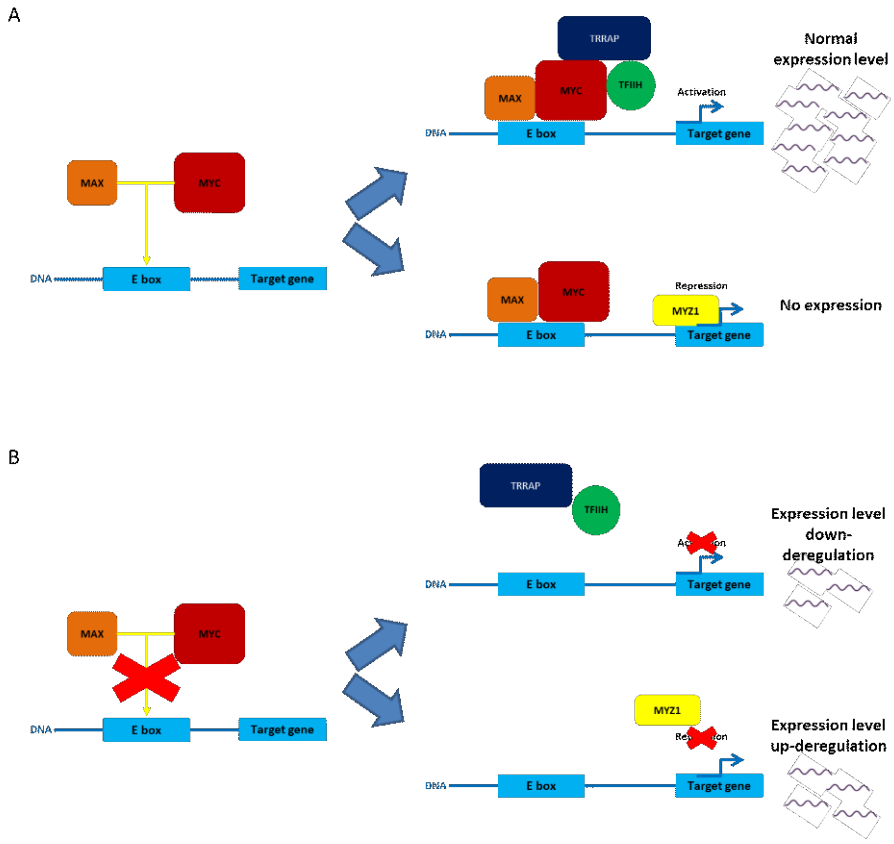


Figure 22. Transcriptional activity of MYC. (A) MYC/Max heterodimer can recruit cofactors to activate (TRRAP, TFIIH) or repress (MYZ1) the transcription. (B) Our hypothesis is that the impairment in MYC/Max complex causes dysregulation in MYC transcriptional activity, disrupting the delicate network and resulting in altered gene expression.

Interestingly, we found down-regulated the lncRNA MINCR which is known to be induced by MYC binding to its promoter region. Therefore, we performed Chromatin Immunoprecipitation assay (ChIP) followed by qPCR in PBMCs of sALS patients and controls. The results showed that MYC binds the TSS region of MINCR less in sALS patients compared to healthy controls (Figure 23). Since MYC binds MINCR promoter to induce its transcription, this alteration can lead to a minor rate of transcription of MINCR, thus leading to MINCR down-regulation in sALS, as we have already demonstrated (Figure 17).

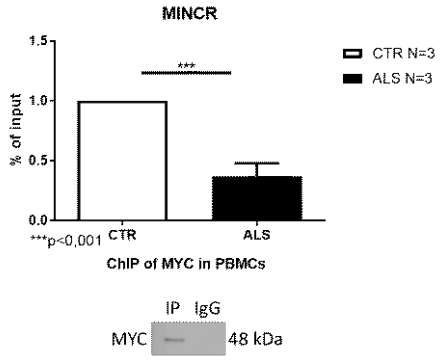


Figure 23. qPCR of MINCR TSS region after ChIP of MYC in PBMCs of sALS patients and control subjects.

6.2 Characterization of MINCR

6.2.1 MINCR and MYC

Considering that many lncRNAs induced by MYC act on MYC itself [197], we hypothesized that the down-regulation of MINCR could alter the MYC network. Therefore, we performed in SH-SY5Y cell line a stable plasmid transfection (SH-MINCR+) and a siRNA-mediated silencing (SH-siMINCR) in order to up- and down-regulate MINCR respectively. As shown in Figure 24, despite an opposite trend in MYC, MYCBP and MAX level in response to up- and down-regulation of MINCR no significative results correlate MINCR expression levels to changes in MYC, MYCBP and MAX expression compared to untreated conditions (SH-MOCK), suggesting that in neuroblastoma cells the mechanisms of action of MINCR does not involve MYC.

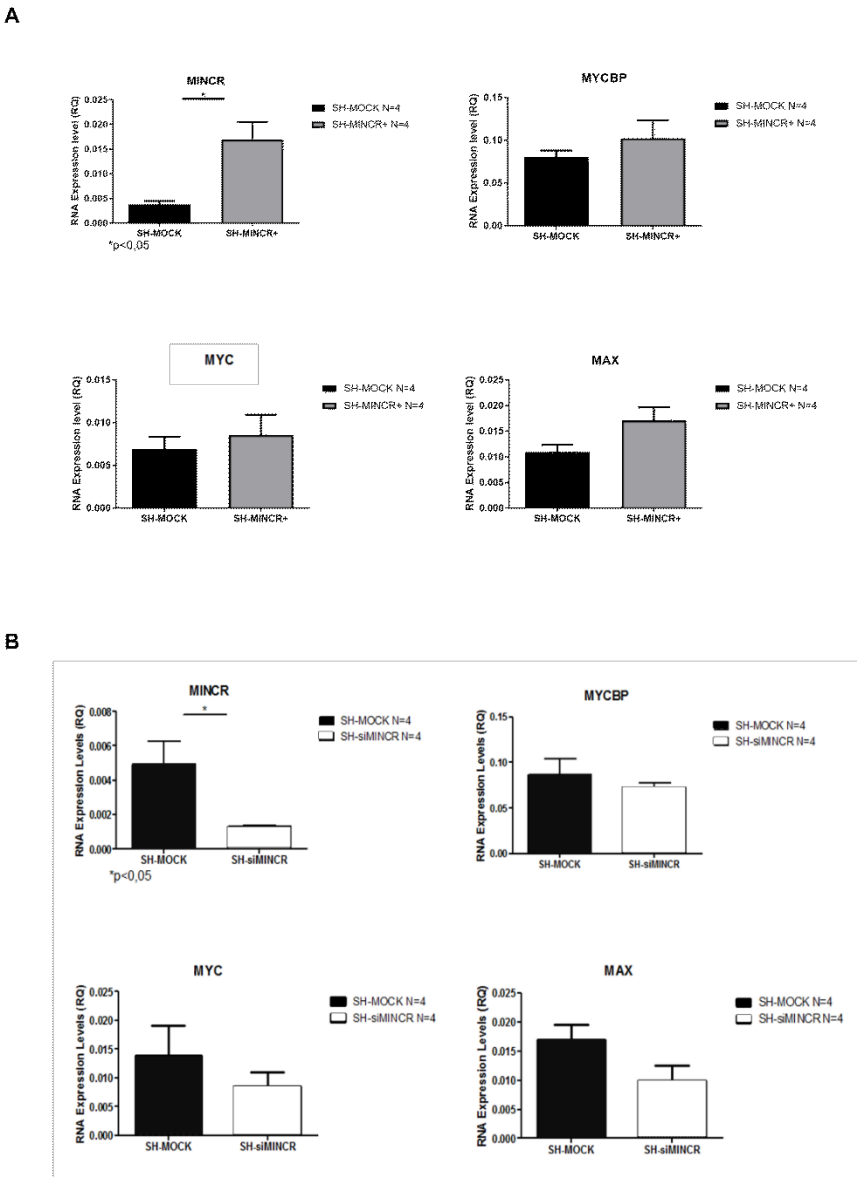


Figure 24. qPCR analysis of MINCR, MYC, MYCBP and MAX in SH-MINCR+ (A) and SH-siMINCR (B) compared to untreated SH-MOCK

Moreover, we investigated also if MINCR could bind directly to MYC in sALS patients. We performed RNA-immunoprecipitation experiments (RIP) of MYC in PBMCs of sALS patients and healthy controls followed by RIP-seq analysis. The NGS data showed that MYC protein and MINCR do not bind directly, while we found other non coding RNAs bound to MYC according to literature. Moreover, Droplet Digital PCR (ddPCR) confirmed the absence of MINCR in immunoprecipitated RNA in both control subjects and sALS patients (Figure 25).

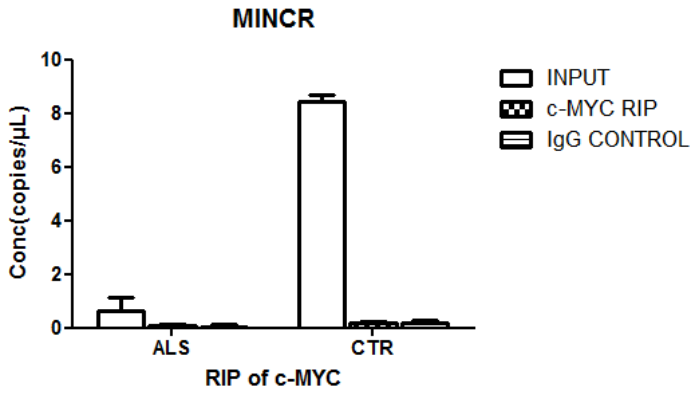


Figure 25. RIP of MYC. DdPCR of MINCR in PBMCs of sALS patients (ALS) and healthy controls (CTR).

6.2.2 MINCR is a nuclear lncRNA

To characterize MINCR, we firstly studied its subcellular localization by Droplet Digital PCR (ddPCR) in cytoplasm and nucleus fraction of PBMCs of sALS and control subjects and in a neuronal cellular model, SH-SY5Y neuroblastoma cell line. All samples showed a predominantly nuclear localization of MINCR according to already published data [198], providing us a suggestion about the compartment of its function and targets (Figure 26).

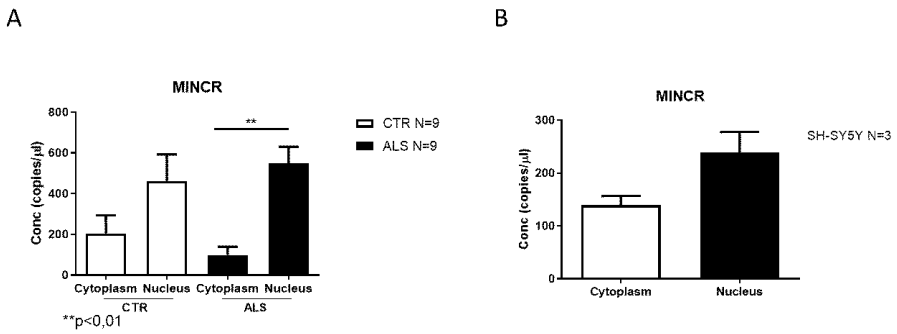


Figure 26. MINCR subcellular localization in PBMCs from (A) ALS patients (ALS) and controls (CTR) and in (B) SH-SY5Y by ddPCR.

6.2.3 Deep sequencing RNA expression profiles in neuronal cell model carrying over- and down-expression of MINCR

To further characterize and investigate the genes and the pathways through which MINCR exerts its effects, we performed a whole transcriptome analysis of SH-SY5Y neuronal cells overexpressing (SH-MINCR+) and down-regulating (SH-siMINCR) MINCR, compared to wild type cells (SH-MOCK).

We firstly characterized the model checking MINCR level and its subcellular localization (Figure 27), showing that overexpression and knockdown of MINCR were successful and that siRNA-mediated silencing and plasmid transfection do not affect its distribution inside the cells.

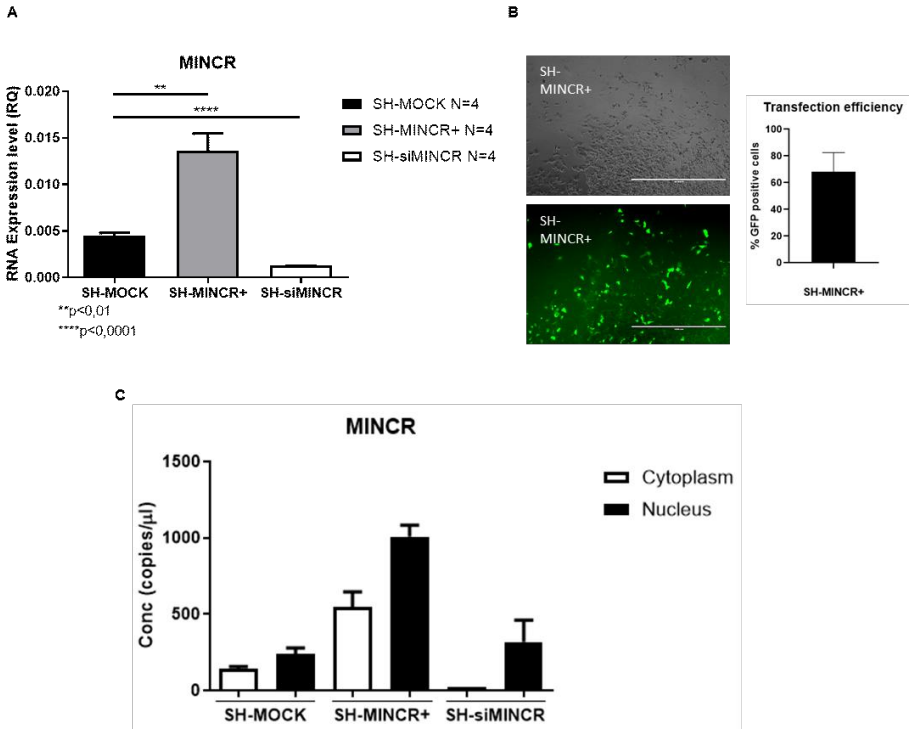


Figure 27. Model characterization: In panel (A), qPCR analysis of MINCR in SH-MINCR+ and SH-siMINCR compared to SH-MOCK. In panel (B), transfection efficiency: bar graph shows the percentage of GFP positive live cells after 10 days of stable transfection. In panel (C), subcellular localization of MINCR in SH-MOCK, SH-MINCR+ and SH-siMINCR.

Principal Component Analysis (PCA) obtained using all differentially expressed genes (DEGs) dataset in SH-MINCR+ and SH-siMINCR compared to SH-MOCK shows different expression profiles, especially highlighting a larger difference between SH-MINCR+ and SH-MOCK (Figure 28A). In order to not manipulate the data we included all the samples in the following analysis, although one of the SH-

MOCK sample is nearer to SH-siMINCR than the others, probably because its MINCR expression levels are lower (RQ of 0.0025 compared to a mean of 0.0044). The volcano plots show the most significant DEGs in SH-MINCR+ and SH-siMINCR compared to SH-MOCK, confirming the different degree of alteration in the two sample types (Figure 28B-C).

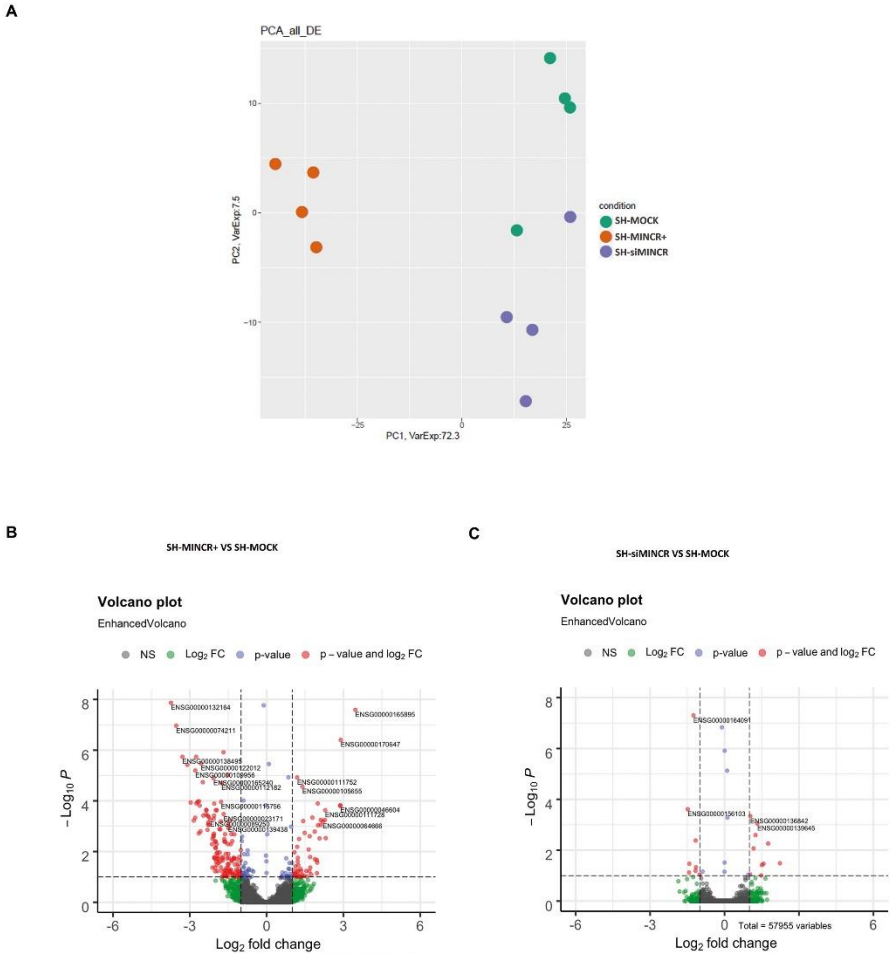


Figure 28. Expression profiles of differentially expressed genes in response to MINCR overexpression and down-regulation. In panel (A), PCA of differentially expressed genes is shown, all comparisons are given between the treated and untreated SH-MOCK cells. In panel (B), volcano plot of differentially expressed genes between SH-MINCR+ and SH-MOCK. In panel (C), volcano plot of differentially expressed genes between SH-siMINCR and SH-MOCK. Gene ID of the most deregulated transcripts are reported, red dots represent differentially expressed genes based on p-value and Fold Change. We considered as differentially expressed only genes showing $|\log2(\text{SH-MINCR+ or SH-siMINCR/SH-MOCK})| \geq 1$ and a False Discovery Rate (FDR) ≤ 0.1.

Indeed, we detected a big difference between the number of DEGs in SH-MINCR+ and SH-siMINCR. A total of 227 DEGs were identified in SH-MINCR+, while in SH-siMINCR we obtained alterations only in 29 genes (Table 7).

	SH-MINCR+		SH-siMINCR	
	mRNAs	lncRNAs	mRNAs	lncRNAs
UP-regulated	57	12	16	1
DOWN-regulated	142	16	10	2
Total	199	28	26	3
	227		29	

Table 7. Number of the statistically significant differentially expressed mRNAs and lncRNAs in SH-MINCR+ and SH-siMINCR compared to SH-MOCK, in terms of up-regulated transcripts, down-regulated transcripts and total deregulated transcripts. Transcripts were considered as differentially expressed when $|\log2(\text{SH-MINCR+ or SH-siMINCR/SH-MOCK})| \geq 1$ and a $FDR \leq 0.1$.

Between the DEGs, the 2 most up-regulated genes are shared between SH-MINCR+ and SH-siMINCR, they are ICAM1 and HMCN1. Both these genes are involved in cellular communication and migration, specifically ICAM1 is a transmembrane protein that stabilizes cell-cell interactions and facilitates leukocyte endothelial transmigration [221] and HMCN1 is an extracellular matrix protein that assembles into fine tracks to organize cell attachment into oriented linear junctions, such as hemidesmosome structure [222]. The presence of shared genes between SH-MINCR+ and SH-siMINCR, especially genes with many functions and thus involved in many different pathways, may suggest that MINCR dysregulation act on a wide range of targets pursuing different undirect mechanisms of regulation that have to be still clarified.

6.2.3.1 DEGs subcellular distribution indicates the MINCR action compartment

Through "The Human Protein Atlas (Cell) - Protein Sub-cellular Localization" network of NDEx, a Cytoscape software plug-in [211], we were able to determine the subcellular distribution of proteins derived from deregulated coding genes in SH-MINCR+ and SH-siMINCR. In both cases the most implicated cellular compartment is the nucleus (Figure 29). In SH-MINCR+ the 54% of DEGs produce proteins localized in the nucleus being part of one or more of the following structures: "Nuclear bodies", "Nuclear speckles", "Nucleoplasm", "Nuclear membrane", "Nucleoli", "Nucleus" and "Nucleoli fibrillar center". The remaining genes are distributed between the other cellular regions, as "Cytosol", "Plasma membrane" and "Mitochondria". We found similar results for SH-siMINCR, where the genes localized in "Nucleus", "Nucleoli", "Nucleoplasm", "Nucleoli fibrillar center" and

"Nuclear bodies" are the 48% of deregulated genes. This analysis is consistent with the subcellular localization of MINCR found in wild-type SH-SY5Y, SH-MINCR+ and SH-siMINCR.

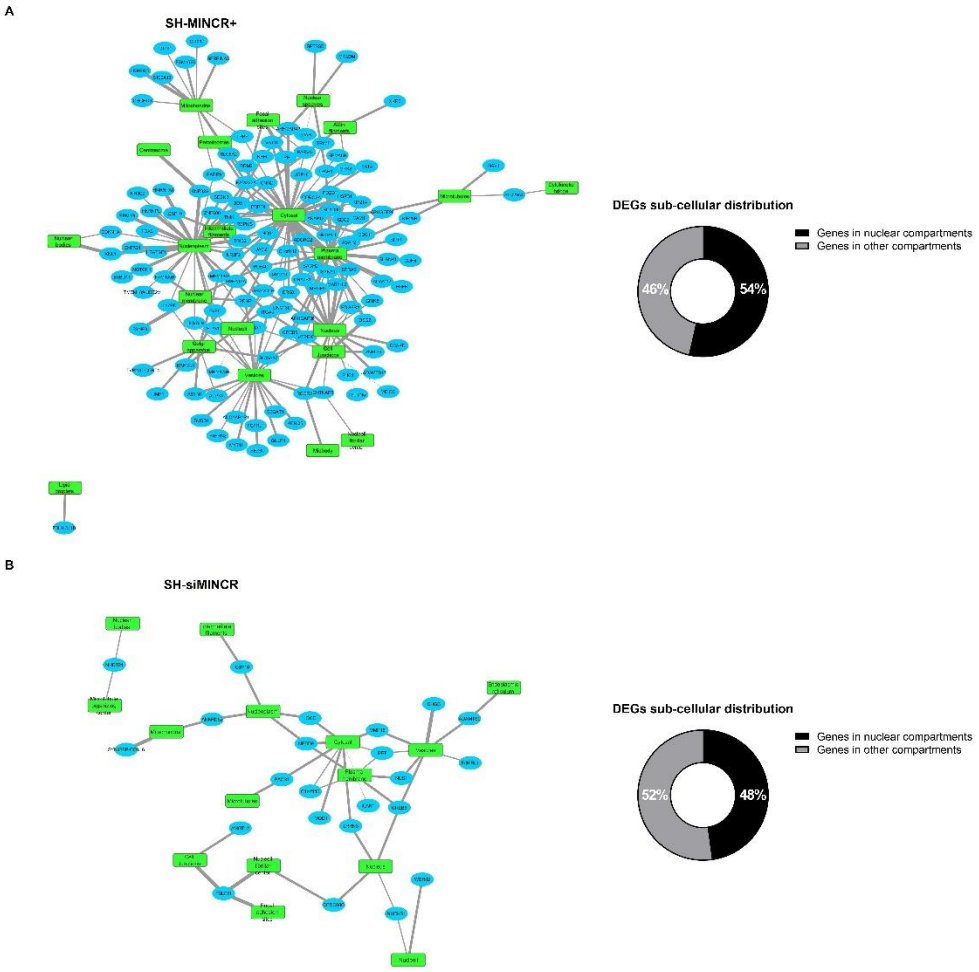


Figure 29. Differentially expressed coding genes subcellular localization. (A) DEGs distribution in SH-MINCR+ and (B) SH-siMINCR from "The Human Protein Atlas (Cell) - Protein Sub-cellular Localization" network of NDEx is shown.

6.2.3.2 GO terms enrichment of deregulated transcripts

Coding gene expression profiles of SH-MINCR+ and SH-siMINCR versus control SH-MOCK were analyzed for GO terms enrichment in Biological Processes, Molecular Function, and Cellular Component (Figure 30).

When considering the top 5 GO terms for SH-MINCR+, in Biological Process we found terms that mainly involved genes of neuronal processes, such as SHANK1 and SHANK2, consisting of almost only down-regulated genes (Figure 30A). In Cellular Component (Figure 30B), we found the main deregulation in "Integral component of plasma membrane" and "Focal adhesion", together with dysregulation of axonal components. Also in these last cases the involved genes were down-regulated. The GO Molecular Function highlights the deregulation in channel and receptor activity (Figure 30C).

On the other hand, the top 5 GO terms in Biological Process in SH-siMINCR highlights terms related to protein structure and extracellular matrix organization (Figure 30D). With respect to Cellular Component (Figure 30E), we found deregulated terms concerning mRNA metabolism involving an epigenetic regulatory complex ("Set1C/COMPASS complex") and the "mRNA cleavage and polyadenylation specificity factor complex" which is a fundamental process in mRNA maturation. The other important alteration regards the up-regulated cytoskeletal remodeling genes, suggesting an altered cytoskeletal modification. We found cytoskeletal abnormalities also in GO Molecular Function, marking again the alteration in this structure (Figure 30F).

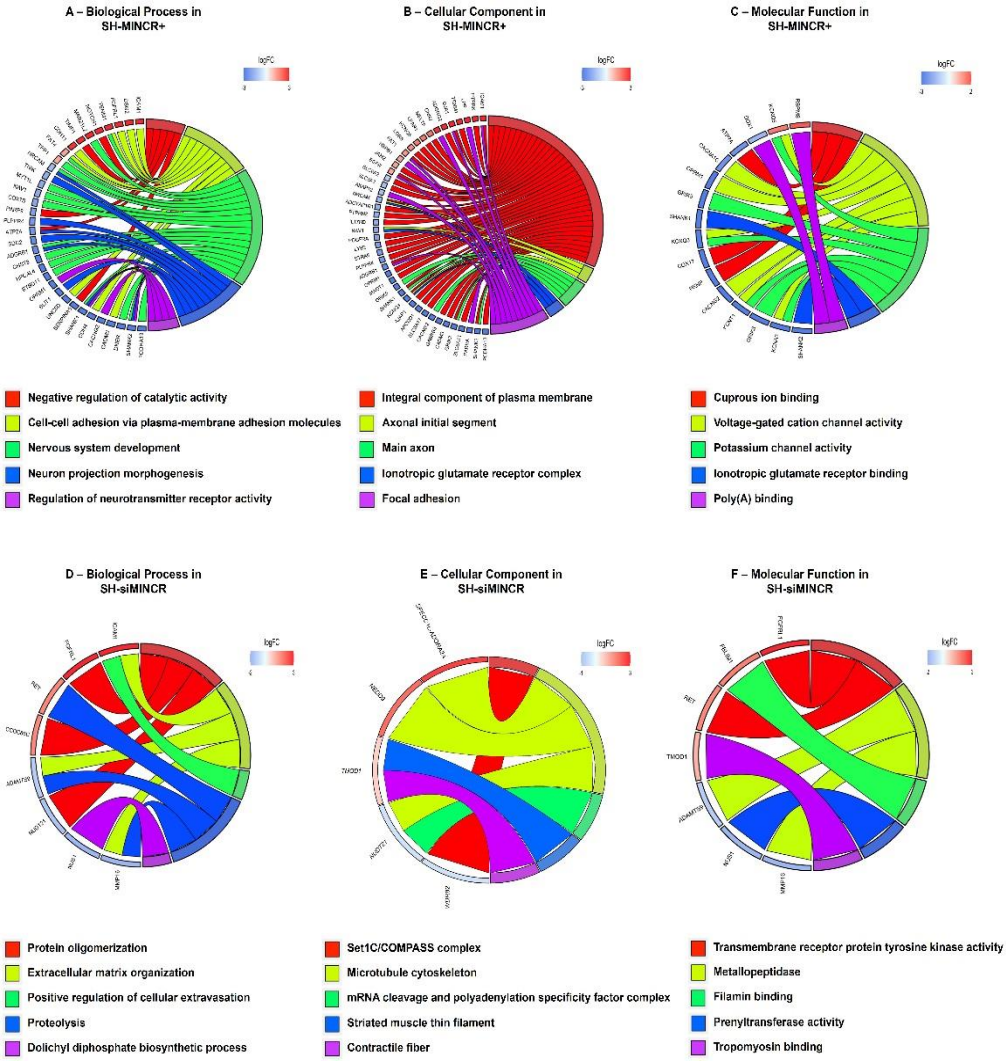


Figure 30. Gene Ontology analysis for coding DEGs in SH-MINCR+ and SH-siMINCR compared to SH-MOCK. Top 5 enriched GO terms for (A) biological process, (B) cellular component and (C) molecular function in SH-MINCR+ and for (D) biological process, (E) cellular component and (F) molecular function in SH-siMINCR are shown. The colors represent different pathways related to the deregulated genes which are displayed in ascendant order for Fold Change.

6.2.3.3 Pathway analysis of deregulated transcripts

The deregulated coding transcripts with a deregulation ≥1 in terms of |Log2FC| were subjected to pathways analysis via the enrichR web tool [209]. We identified the

deregulated pathways in SH-MINCR+ and SH-siMINCR with KEGG 2019 and Wikipathways tools (Figure 31).

According to literature, the overexpression of MINCR is implicated in pathways involved in tumour onset and progression. The KEGG and Wikipathways analysis support this data even in a neuronal context, as in the KEGG 2019, amongst the top 20 deregulated pathways (Figure 31A), we found 5 pathways implicated in cancer mechanisms. These pathways are: “Prostate cancer”, “p53 signaling pathway”, “Melanoma”, “Glioma” and “Bladder cancer”. Wikipathways analysis (Figure 31B) also showed tumour implication: “Pathways regulating Hippo signaling”, “Hippo-Merlin signaling dysregulation” and “Deregulation of Rab and Rab effector genes in bladder cancer”.

Because of the small number of DEGs in SH-siMINCR, the emerged pathways from KEGG and Wikipathways analysis were not statistically significant, but they highlighted different alterations compared to SH-MINCR+. Indeed, the most implicated pathways concern RNA metabolism and inflammation (in Figure 31C only pathways with 2 involved genes are shown). In particular, “mRNA surveillance pathway”, “TNF signaling pathway” and “NF-kappa B signaling pathway” result deregulated in KEGG analysis. Wikipathways analysis reports mRNA processing deregulation and a more considerable implication of inflammation since 8 out of 20 deregulated pathways are involved in inflammatory mechanisms.

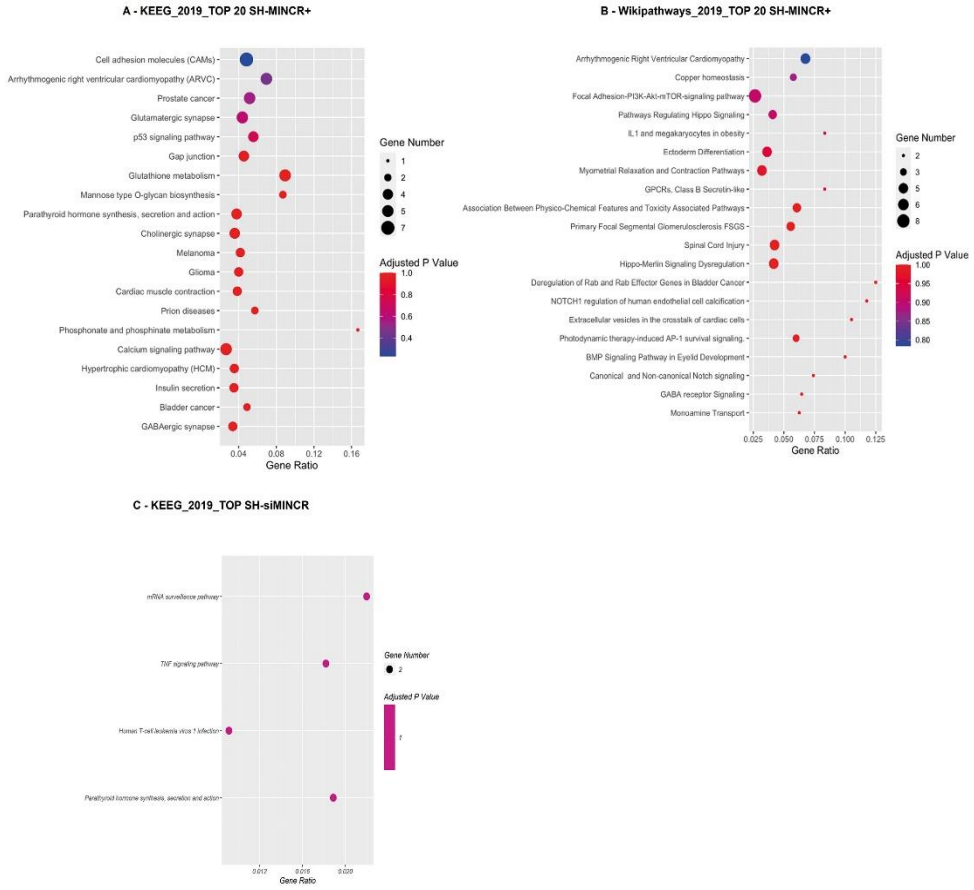


Figure 31. Pathway analysis for coding DEGs in SH-MINCR+ and SH-siMINCR compared to untreated SH-MOCK. The y-axis represents the name of the pathway, the x-axis represents the Rich factor, dot size represents the number of different genes and the color indicates the adjusted p-value. (A) Dot plot of top 20 deregulated pathways in SH-MINCR+ from KEGG analysis. (B) Dot plot of top 20 deregulated pathways in SH-MINCR+ from Wikipathways analysis. (C) Dot plot of deregulated pathways with 2 involved genes in SH-siMINCR from KEGG analysis.

6.2.3.4 MINCR transcript levels impact on oncogenesis

Since it has been demonstrated that MINCR acts in oncogene pathways in different types of cancer, we evaluated its impact on tumorigenic genes and pathways in neuroblastoma cells. Using "The Human Protein Atlas (Pathology) – Scored Gene-Cancer Correlation" network of NDEx app [211], we were able to correlate deregulated coding genes and tumour. The networks report the correlation between mRNA expression level and patient's survival for several cancer types. As shown in

Figure 32A-32B, 103 out of 199 coding genes emerged as cancer related in SH-MINCR+ and 12 out of 26 coding genes in SH-siMINCR.

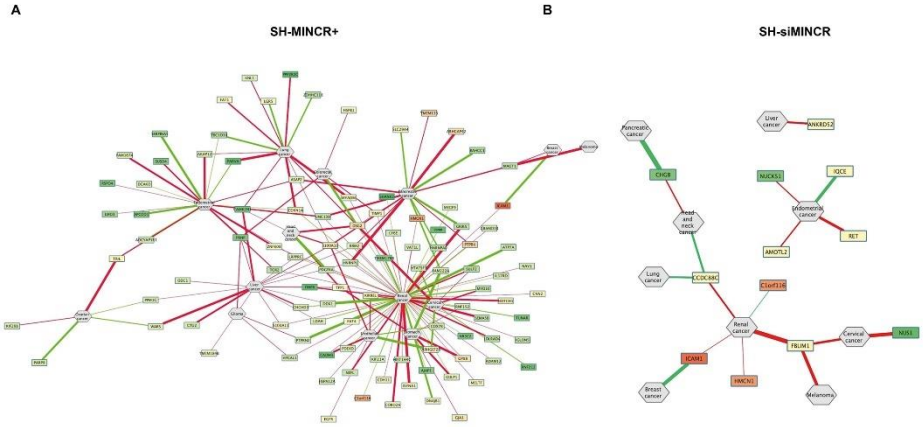


Figure 32. MINCR-cancer correlation. DEGs associated to cancer in (A) SH-MINCR+ and (B) SH-siMINCR performed through "The Human Protein Atlas (Pathology) – Scored Gene-Cancer Correlation" network are shown. The network shows the correlation between mRNA expression level and patient's survival, the edge color represents prognostic information (green=favourable, red=unfavourable) while edge weight the correlation score.

In SH-MINCR+ the most deregulated cancer-related genes are involved in cell-signaling, cell-cell contact and in oxidative stress metabolism. PTPRK, DSG2 and GPX8 are the most up-regulated genes. PTPRK is a tyrosine phosphatase enzyme involved in a variety of cellular process including cell growth, mitotic cycle and tumorigenic transformation [223]. DSG2 encodes for the desmoglein-2 protein which is localized to desmosomes and functions to adhere adjacent cells together [224]. GPX8 is part of the glutathione peroxidase family and has the ability to reduce free hydrogen peroxide in order to protect the cell form oxidative stress-induced damage [225]. With the exception of DSG2 in colorectal cancer where its presence influences positively the cancer status, the network shows that the up-regulation of these genes leads to worse prognosis in pancreatic cancer (PTPRK), head and neck cancer and cervical cancer (DSG2) and stomach and renal cancer (GPX8), thus confirming an oncogenic role for MINCR overexpression. Moreover, the network reports as favourable the presence of genes that are strongly down-regulated in SH-MINCR+, such as SHANK2 in renal cancer and PARVA in endometrial cancer. SHANK2 is involved in postsynaptic density organization in excitatory neurons, but it is present also in other tissues where it localizes with the cyclic nucleotide phosphodiesterase PDE4D precluding cAMP/PKA signals [226], interestingly also PDE4D is down-regulated in SH-MICNR+. PARVA is an actin-binding protein associated with focal contacts and plays a role in cell adhesion, motility and survival [227], it is also been associated to lung cancer as an unfavourable prognostic factor. Interestingly, since

we are studying a neuronal model, “Glioma” appeared to be a cancer promoted by MINCR overexpression.

In SH-siMINCR there is less tendency to tumorigenicity compared to SH-MINCR+. Indeed, as it is shown by the network (Figure 5B), between the most deregulated genes there are down-regulated factors that are usually up-regulated in cancer and vice versa. For example, CCDC88C, a negative regulator of the Wnt signalling pathway [228], is up-regulated in response to MINCR down-regulation leading to better outcome in lung cancer and head and neck cancer, but it is also an unfavourable gene in renal cancer. NUS1 encodes for a subunit of cis-prenyltransferase and is essential for dolichol synthesis and protein glycosylation [229], in the network it is reported as a disadvantageous gene in cervical cancer, but it is down-regulated in SH-siMINCR. Like NUS1, also NUCKS1, which presence is negative in endometrial cancer due to its action on DNA repair mechanism [230], is down-regulated after MINCR silencing depicting an opposite scenario compared to tumour. Table 8 resumes the above-mentioned genes and their impact in cancer diseases based on their fold-change deregulation.

		DEGs	Prognosis	Disease
SH-MINCR+	**Upregulated**	PTPRK		Pancreatic cancer
		DSG2		Head and neck cancer
				Cervical cancer
				Colorectal cancer
		GPX8		Stomach cancer
				Renal cancer
	Down-regulated	SHANK2		Renal cancer
		PARVA		Endometrial cancer
SH-siMINCR	**Upregulated**	RET		Endometrial cancer
		CCDC88C		Renal cancer
				Lung cancer
				Head and neck cancer
	Down-regulated	NUS1		Cervical cancer
		NUCKS1		Endometrial cancer

Table 8. Favourable and unfavourable genes in cancer based on their fold-change in response to MINCR up- and down-regulation. Red: unfavourable prognosis, Green: favourable prognosis.

The difference in tumorigenic potential of MINCR up- and down-regulation can be observed also from KEGG pathways analysis: 26 pathway out of 160 (16.25%) are related to cancer in SH-MINCR+, while only 5 out of 53 (9.4%) in SH-siMINCR (Figure 33).

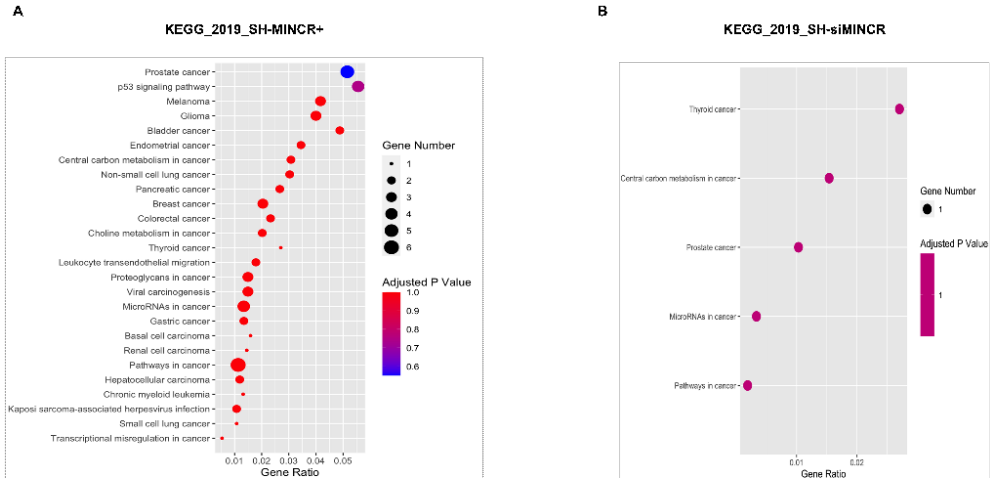
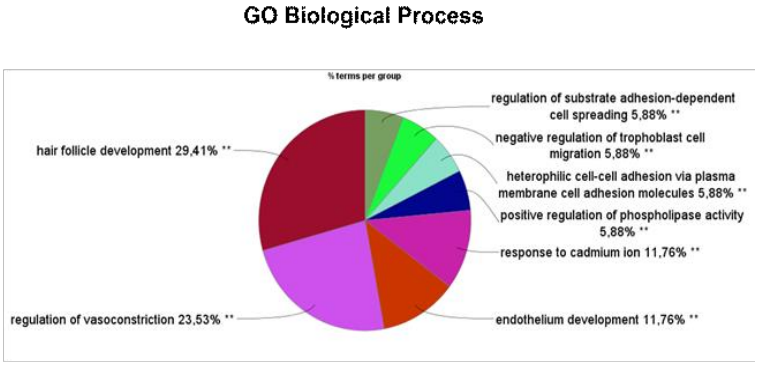


Figure 33. MINCR-cancer correlation. Dot plot of cancer-related pathways from KEGG analysis in (A) SH-MINCR+ and (B) SH-siMINCR. The y-axis represents the name of the pathway, the x-axis represents the Rich factor, dot size represents the number of different genes and the color indicates the adjusted p-value.

Finally, the mechanisms exerted by the deregulated cancer-related genes in SH-MINCR+ and SH-siMINCR are different. We performed GO analysis of biological process with ClueGO app for Cytoscape [212] on cancer-related genes from "The Human Protein Atlas (Pathology) – Scored Gene-Cancer Correlation" network (Figure 34A-34B). Due to the small number of DEGS in SH-siMINCR we were able to also perform a manual research for gene-cancer association, the resulted genes were included in GO analysis. Biological processes associated with MINCR overexpression (SH-MINCR+) mostly concern cell-cell communication and motility. In SH-siMINCR GO analysis especially highlights the importance of RNA metabolism and transcription modulation, showing different biological processes compared to SH-MINCR+.

A



B

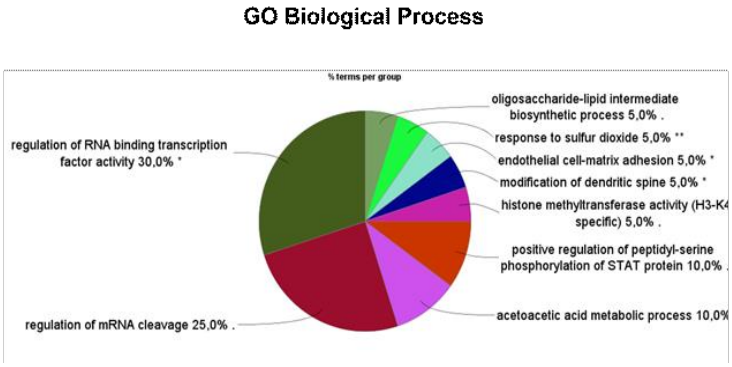


Figure 34. GO biological process analysis of cancer-related genes in (A) SH-MINCR+ and (B) SH-siMINCR using ClueGO Cytoscape app are shown. Data are displayed as % terms per group, * indicates term with p-value <0.05 and ** p-value <0.01

6.2.3.5 MINCR transcript levels impact on neurodegeneration

Since it has been demonstrated that numerous lncRNAs act in more than one type of pathologies implementing different mechanisms, we wanted to elucidate MINCR possible pathways in other diseases. Giving that the only available data about MINCR beyond cancer are about neurodegenerative disorders [86] [205], we performed DEGs analysis through DisGeNET platforms [213]. As shown in Figure 35A-35B, 48 out of 199 coding genes emerged as involved in neurodegenerative diseases in SH-MINCR+ and 5 out of 26 in SH-siMINCR.

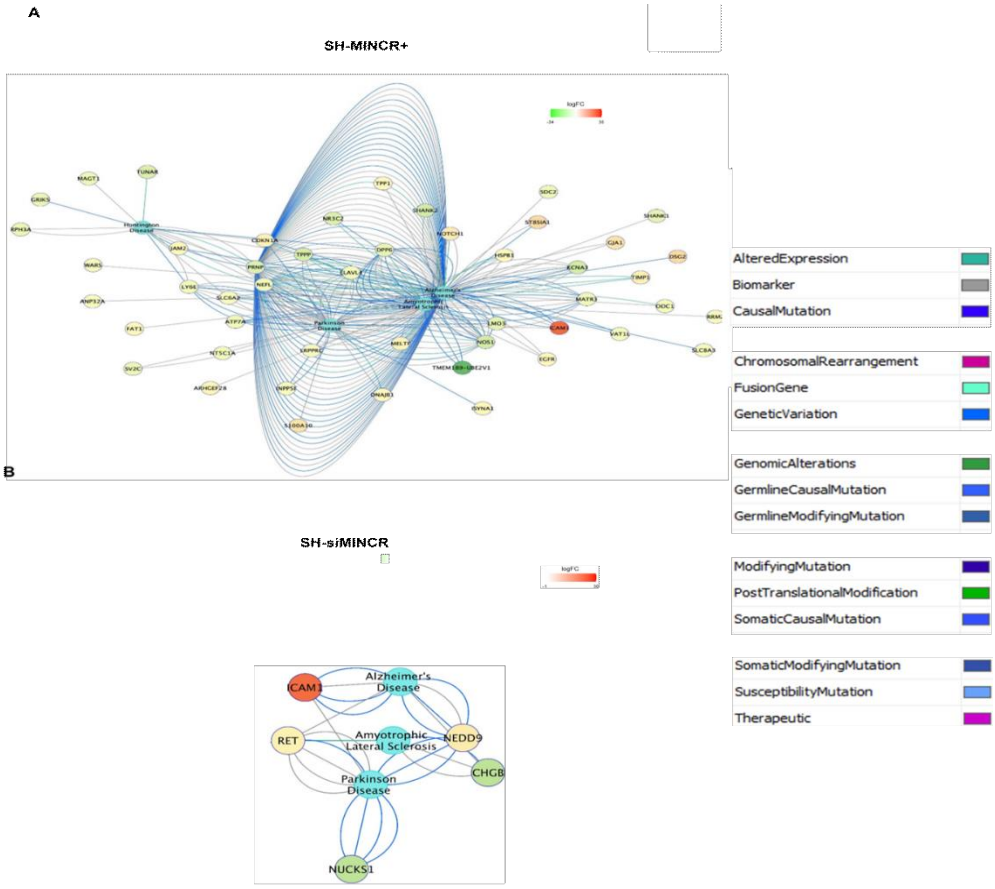


Figure 35. MINCR-neurodegeneration correlation. DEGs associated to neurodegenerative diseases in (A) SH-MINCR+ and (B) SH-siMINCR performed through DisGeNET database are shown.

In SH-MINCR+ the most deregulated genes are related to Alzheimer's disease. ST8SIA1 is up-regulated in response to MINCR overexpression and encodes for GD3 synthase which is responsible for biosynthesis of the b- and c-series gangliosides. In Alzheimer's disease mouse model, the lack of GD3 inhibits Aβ-induced cell death and Aβ aggregation [231]. DSG2 rs8093731 variant has been classified as a top 20 risk variants in Alzheimer's disease with brain amyloidosis and it seems to have an association with amyloid deposition [232]. Two of the most down-regulated genes in response to MINCR overexpression are associated to Alzheimer's disease, but, on the contrary, they have been found to be up-regulated in the disease. SHANK2, a scaffold protein of glutamate excitatory synapses, is present at high levels leading to disruption of glutamate receptors in Alzheimer disease [233]. The second gene is KCNA3, a voltage-gated potassium channel, which is highly expressed on microglia during Alzheimer's disease and promotes neuroinflammation, production of reactive oxygen species and amyloid-mediated microglial priming [234]. Also NOS1, neuronal NO synthase, is one of the most down-regulated genes associated to

neurodegeneration inside the network. A repeat in promoter region of NOS1 has been associated with Alzheimer's disease [235], a variation in exon 28 and 29 has been associated to Parkinson's disease [236] and NOS1 up-regulation has been demonstrated in reactive astrocytes of Amyotrophic Lateral Sclerosis human spinal cord [237].

In SH-siMINCR the up-regulated genes are RET and NEDD9. RET has been implicated in Amyotrophic Lateral Sclerosis where its decrease in human spinal cord motor neurons has been proposed as compensatory mechanism for preventing motor neuron degeneration and its increase in microglial cells in spinal cord of G93A mouse has been described [238]. Moreover, high level of RET in microglia has been observed also in Parkinson's disease [239]. NEDD9 is involved in the outgrowth of neurites and polymorphisms and variations of this gene have been associated to Alzheimer's and Parkinson's disease [240]. Up-regulation of NEDD9 has also been described in monocytes of rapidly progressive Amyotrophic Lateral Sclerosis patients [241]. The down-regulated genes associated to neurodegenerative diseases are CHGB and NUKS1. CHGB encodes a tyrosine-sulfated secretory protein, member of granin family, whose down-regulation in motor cortex of ALS patients leads to a reduction of glycolytic energy supply that constitutes one of the major causes for neuronal cell death [242]. Finally, after MINCR down-regulation also NUCKS1 is down-regulated and its decrease has been described in Parkinson's disease [243]. Taken together these data suggest that the down-regulation of MINCR could lead to a neurodegenerative phenotype more than MINCR up-regulation. In Table 9 DEGs and their impact in neurodegenerative disorders based on their fold-change deregulation are summarized.

	SH-MINCR+	DEGs	Prognosis	Disease
SH-MINCR+	**Upregulated**	ST8SIA1		AD
		DSG2		AD
	Down-regulated	SHANK2		AD
		KCNA3		AD
		NOS1		AD
				PD
				ALS
SH-siMINCR	**Upregulated**	RET		ALS
				PD
		NEDD9		AD
				PD
				ALS
	Down-regulated	CHGB		ALS
		NUCKS1		PD

Table 9. Favourable and unfavourable genes in neurodegeneration based on their fold-change in response to MINCR up- and down-regulation. Red: unfavourable prognosis, Green: favourable prognosis.

We performed GO analysis for biological process with ClueGO app for Cytoscape [212] of neurodegenerative-related DEGs from DisGeNET analysis (Figure 36A-B). We included in GO analysis also the genes associated to neurodegeneration obtained from manual research of DEGs in SH-siMINCR, as we did for cancer-related genes. Mechanisms exerted by DEGs in SH-MINCR+ linked to neurodegeneration concern mainly dendrite structure, the organization of synaptic density and neurotransmission and the response to copper ion. Different processes emerged from SH-siMINCR analysis, the most involved are the modulation of transcription and the response to sulfur dioxide, a toxic gas present in air pollution that has been associated to the onset of neurodegenerative diseases [244].

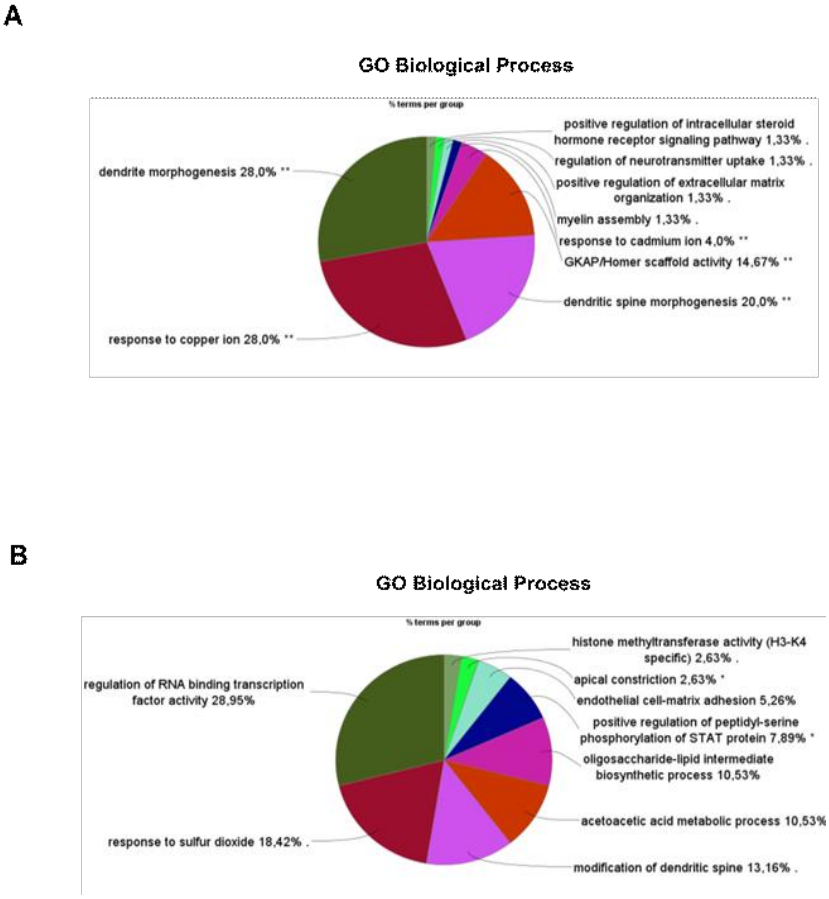


Figure 36. GO biological process analysis of neurodegeneration-related genes in (A) SH-MINCR+ and (B) SH-siMINCR using ClueGO Cytoscape app are shown. Data

are displayed as % terms per group, * indicates term with p-value <0.05 and ** p-value <0.01

6.3 MINCR and ALS

6.3.1 MINCR binds to histone H1 in differentiated SH-SY5Y

Firstly, we focused on identifying specific MINCR physical interactors. To that end, we performed RNA pull-down using in vitro transcribed MINCR RNA or an unrelated RNA of similar length (the antisense sequence) as control. The RNAs were incubated with nuclear extract of differentiated SH-SY5Y cells and mass spectrometry (MS) was performed on the differential bands found on the retained proteins (Figure 37). Histone H1 was identified as a protein bound to MINCR with 4 unique peptides for HIST1H2AH, 19 unique peptides for HIST1H1C, 22 unique peptides for HIST1H1B, 6 unique peptides for HIST1H1A and 9 unique peptides for H1FX.

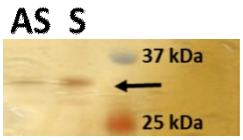


Figure 37. Silver stained gel with the proteins retained in the RNA pull-down experiment by MINCR (S) and antisense (AS) as negative control, the differential band analyzed by mass spectrometry is indicated by an arrowhead.

6.3.2 MINCR knock-out leads to alteration in cytoskeleton and increase in late apoptosis

In order to investigate the effect of MINCR in ALS, we knocked-out MINCR by CRISPR-Cas9 system in SH-SY5Y cells. After screening of the clones, we chose a clone with deletion in the aimed region confirmed by PCR with two pairs of primer designed specifically for deleted and non-deleted region and by qPCR (Figure 38A-B-C).

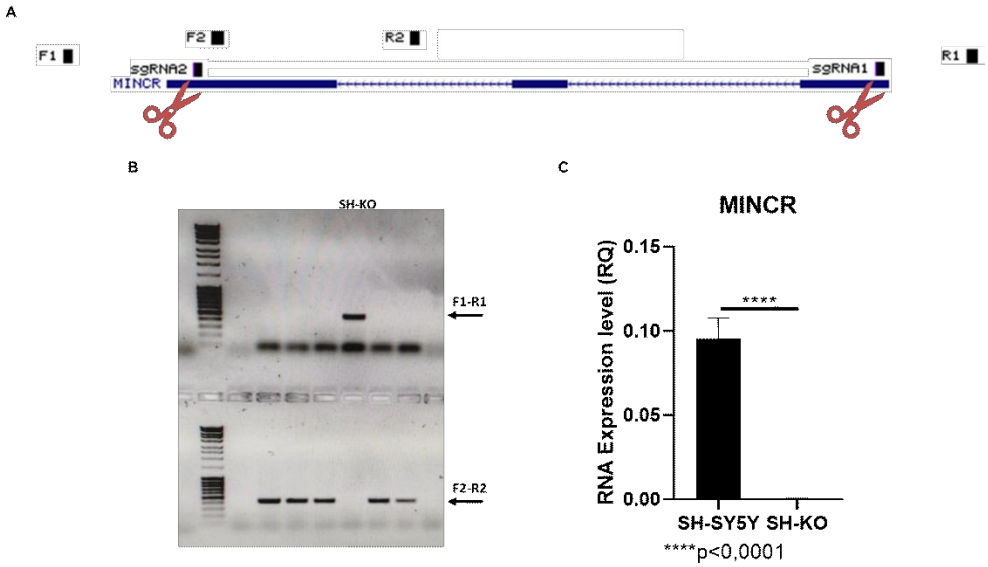


Figure 38. (A) Schematic representation of CRISPR-Cas9 technology to delete the MINCR gene and annealing sites of primers to detect deleted and non-deleted regions on RNA. (B) PCR validation of deleted region with two different pairs or primer. (C) Relative RNA expression level of MINCR for wild-type (SH-SY5Y) and deleted clone (SH-KO)

From a morphological point of view, SH-KO showed a different shape and characteristics compared to wild-type SH-SY5Y. They lost the neuroblast-like cells phenotype, being smaller and more curved, and showed cell shrinkage, membrane bleb formation, detachment from the surface and aggregation, which are all hallmarks of apoptosis (Figure 39).

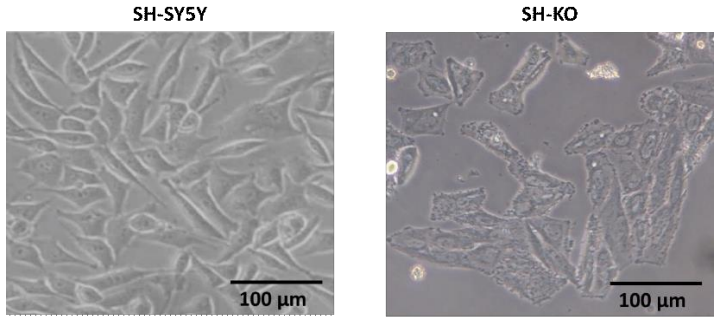


Figure 39. Bright-field images of undifferentiated SH-SY5Y and SH-KO

Consistent with these observations, the only available data about MINCR knock-down revealed an impact on apoptosis [198]. Therefore, we performed apoptosis assay through Annexin V and 7-AAD staining on differentiated wild-type SH-SY5Y

and differentiated SH-KO. As we expected, we found a high percentage of Annexin V+ cells also in wild-type SH-SY5Y samples caused by the differentiation protocol [245] [246]. Nevertheless, the results showed that MINCR knock-out increases apoptosis in SH-SY5Y cells, specifically late apoptosis (Figure 39A-B). Indeed, the percentage of apoptotic cells (including only Annexin V+, only 7-AAD+ and double Annexin V+/7-AAD+ cells) is almost doubled in SH-KO cells (Figure 40A), but the major difference is detectable in double Annexin V+/7-AAD+ cells, which represent cells in late apoptosis (Figure 40B).

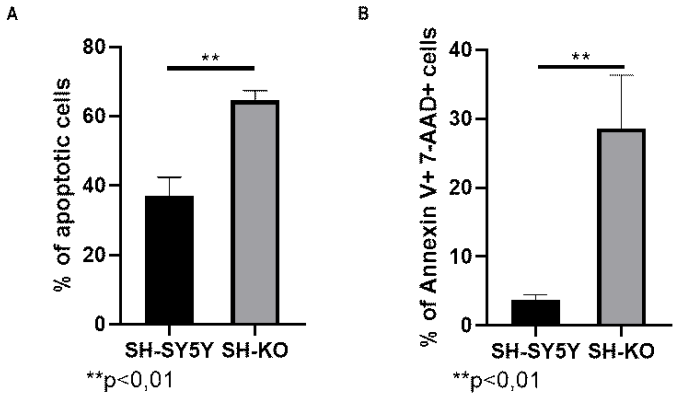


Figure 40. (A)Percentage of apoptotic cells (only Annexin V+, only 7-AAD+ and double Annexin V+/7-AAD+ cells). (B) Percentage of late apoptotic cells (double Annexin V+/7-AAD+ cells)

Furthermore, SH-KO lost the ability to generate neurites in response to differentiation protocol (Figure 41), showing an impairment in structures fundamental for shape and internal organization, such as the cytoskeleton.

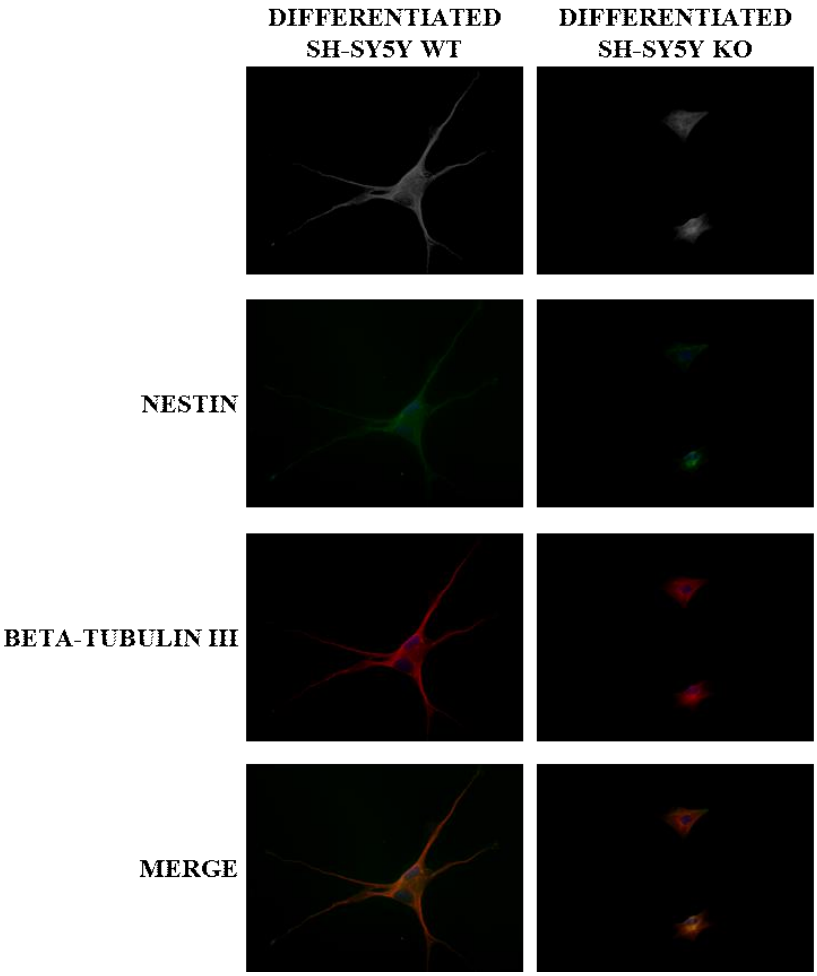


Figure 41. IF of Nestin and Beta-Tubulin III in differentiated wild-type SH-SY5Y and differentiated SH-KO

7. DISCUSSION

In more than 90% of cases, ALS presents in a sporadic form, meaning without familial history and without being associated to a known genetic risk. Moreover, even if various pathogenic mechanisms, which could be implicated in neuronal death, have been identified, none has been defined as causative for ALS. Considering the molecular aspects, in recent years, RNA metabolism has captured more and more researchers' attention, both involving coding and non coding transcripts. Dysregulation in RNA-binding proteins, in gene expression and in transcription factor activity have emerged, beside the idea that alterations in lncRNAs play an important role in ALS pathogenesis. Despite the numerous studies that have addressed this topic, alterations in RNA metabolism, especially concerning transcriptional deregulation and non coding RNAs, remain unclear.

In this context, we performed a whole transcriptome analysis in PBMCs of sALS patients compared to healthy controls that revealed two main concepts: a deregulation in genes involved in transcription and a significative alteration of lncRNA expression [86]. In the light of this considerations, we found interesting the deregulation of MYCBP and MINCR RNA levels in sALS, both related to the transcription factor MYC.

MYC oncogene acts as a central hub of the cell, integrating signals from numerous pathways to control gene expression programs and regulate many biological functions, including cell growth, proliferation, apoptosis, differentiation, and transformation [177]. MYC works only in complex through the C-terminal and the N-terminal domain that binds Max and MYCBP, respectively. When bound to DNA, the MYC-Max heterodimer recruit complexes that modify chromatin [175], while MYCBP stimulates the activation of E-box-dependent transcription by MYC [176]. MYC and its molecular partners have been implicated in the onset of neurodegenerative disorders, such as AD [190] and PD [193].

Starting from our whole transcriptome analysis in sALS patients, we decided to investigate MYC network. Firstly, we confirmed RNA-seq data through qPCR both in PBMCs and in spinal cord tissue, which is specific for ALS, confirming significant decrease of MINCR and MYCBP in sALS patients compared to healthy controls, showing higher MYC levels in patients compared to controls, while Max did not show a significant variation. So, these data demonstrated that, unexpectedly, there is an alteration in MYC and MYC-related genes expression in sALS.

To understand if this alteration could have an impact on MYC target gene expression, we analyzed RNA-seq data of sALS patients and controls and found out that 75 coding DEGs out of 87 (86.2%) resulted to be MYC targets. The list includes both down-regulated and up-regulated genes, and this is not surprising since, beside the well-known activity of MYC as transcriptional activator, it can act suppressing gene

transcription too as heterodimer with Max and interacting with other co-factors (proteins or non coding RNAs). These data suggested a great alteration in MYC network expression that is reflected in global gene expression alteration, lending a potentially central role for MYC in RNA metabolism dysregulation of sALS.

For these reasons, we decided to investigate MYC network. First of all, we studied protein levels of MYC, Max and MYCBP in PBMCs of sALS patients and controls showing that protein levels were similar without statistically differences, thus suggesting that the possible role of MYC in ALS deregulation doesn't lie in protein levels alteration. Therefore, we assessed the transcriptional activity of MYC investigating the heterodimer formation with Max. We revealed that in sALS MYC and Max binding is minor than in control subjects, showing that the MYC/Max heterodimer formation is impaired. The different binding between MYC and Max in sALS patients compared to controls could be due to MYC altered subcellular distribution in sALS patients. Indeed, MYC to exerts its function is mainly localized in the nucleoplasm where it dimerizes with Max [218]. In PBMCs of sALS patients we found, on the contrary, high MYC levels in cytoplasm and low MYC levels in nucleus. Mislocalization of MYC and other transcription factors have already been reported as a key event in altering proper protein function resulting in dysregulation [219] [220].

Taken together these first part of data suggested that in sALS there is an alteration in MYC network, probably caused by the mislocalization of MYC protein inside the cell that hinders the normal formation of MYC/Max transcriptional active complex. The impairment in heterodimer formation could be the cause of the great alteration in RNA expression level of many MYC target genes, making MYC a central factor in RNA metabolism dysregulation in ALS.

Ttranscriptome profiling of tumor samples has shown that many lncRNAs can be differentially regulated by MYC [197]. About this point, Doose and collaborators [198] investigated the extent of changes that MYC can induce in lncRNA expression. The intersection of data from two different cell line model carrying a MYC-inducible construct and sequencing data from Burkitt Lymphoma patients' samples led to the identification of the MYC-induced long non-coding RNA (MINCR). The MINCR gene is an intergenic lncRNA located between two coding genes GLI4 and ZNF696 on chromosome 8q24.3. In MYC-positive lymphoma samples, MINCR showed a high positive correlation with MYC mRNA expression level and its promoter region is bound by MYC protein at the transcription start site [198]. Moreover, MINCR is overexpressed and correlates with poor prognosis in other types of cancer, such as gallbladder cancer [199], hepatocellular carcinoma [200], non small-cell lung cancer [201], oral squamous cell carcinoma [202], nasopharyngeal carcinoma [203] and glioma [204]. MINCR knockdown experiments demonstrated that it is able to control the expression level of a set of MYC target genes involved in cell cycle initiation and tumour progression [198], but its mechanism of action remains unclear. We found MINCR down-regulation in sALS patients, thus we firstly investigated the cause of its decrease. Through a ChIP assay followed by qPCR, we demonstrated that MYC

binds the TSS region of MINCR less in sALS patients compared to healthy controls. Since MYC binds MINCR promoter to induce its transcription and since we demonstrated that there is an impairment in MYC/Max complex, we suggest that this alteration can lead to a minor rate of transcription of MINCR, thus leading to MINCR down-regulation in sALS. Given that many MYC regulated lncRNAs are able to control the expression of MYC itself acting both at transcriptional and post-transcriptional levels [197], we evaluated the possible regulatory mechanism exerted by MINCR on MYC network. We demonstrated that neither the overexpression nor the knockdown of MINCR in SH-SY5Y neuroblastoma cell line caused a significative alteration in MYC, MYCBP and Max mRNA expression compared to untreated conditions. Moreover, RIP of MYC in PBMCs of sALS patients and control subjects followed by RIP-seq and ddPCR analysis showed that MINCR does not bind to MYC protein, led us to conclude that the mechanisms of action of MINCR does not involve MYC directly.

RNA-sequencing analysis of SH-SY5Y cells overexpressing and silenced for MINCR allowed us to identify different deregulated RNAs. Generally, the MINCR overexpression caused an up-regulation of cancer genes and a down-regulation of genes implicated in the central nervous system. On the other hand, the down-regulation of MINCR led to an up-regulation of genes implicated in RNA metabolism, a fundamental cellular process involved in neurodegenerative disease pathogenesis. RNA sequencing pointed out a larger RNA perturbation in response to MINCR up-regulation (227 DEGs) compared to MINCR down-regulation (29 DEGs). Since the plasmid overexpression and the siRNA-mediated silencing both changed MINCR expression levels with a 3X factor, these data suggest a major role in multiple disruption for the overexpression of MINCR involving many different genes and pathways, while it seemed that MINCR down-regulation leads to smaller but more specific alterations. Through the analysis of subcellular localization of DEGs derived proteins, we found that changes in MINCR expression levels cause alteration mainly in nuclear compartments in both cellular models. This was consistent with the distribution of MINCR in SH-SY5Y cells, where it predominantly localizes into the nucleus. The localization of lncRNAs could be informative about the role and the types of mechanism carried out by an RNA transcript. Nuclear lncRNAs contribute to several biological processes, including regulation of gene expression and nuclear organization [247]. Intriguingly, some of the nuclear DEGs in SH-MINCR+ and SH-siMINCR are DNA binding proteins, such as zinc finger protein family members [248] (ZNF536, ZNF662, ZNF521, ZNF536 and ZNF608) deregulated in SH-MINCR+, NUCKS1 [230] in SH-siMINCR and the CAMP Responsive Element Binding Protein 5 (CREB5) [249] in both cell models. Through KEGG 2019, Wikipathways and GO enrichment analysis we were able to obtain a detailed profile of the pathways altered by MINCR expression level. The different outcomes caused by up-regulated and down-regulated MINCR suggest that the expression level can be a regulator of lncRNA activity. Indeed, according to literature, processes involved in cancer were the main enriched pathways in response to MINCR overexpression (“Prostate cancer”, “p53 signaling pathway”, “Melanoma”, “Glioma” and “Bladder cancer”), related to growth factor signaling

(EGFR and PDGFR) and cell cycle (CDKN1A). Interestingly, CDKN1A is upregulated in SH-MINCR+ and it is an unfavourable factor in the types of cancer emerged from pathway analysis: in prostate cancer CDKN1A overexpression is associated to worst clinical outcome [250], in gliomas CDKN1A expression is an indicator of shortened disease-free survival [63] and in bladder cancer CDKN1A is associated to invasiveness [251]. On the other hand, in response to MINCR down-regulation, RNA metabolism and inflammation were the main altered pathways, highlighting two new MINCR related processes. In GO analysis the down-regulation of MINCR also leads to the dysregulation of cytoskeleton, a critical structure for neurons. From that GO enriched terms, NEDD9 emerged as a deregulated gene involved in microtubule network stabilization and it is worthy to note that NEDD9 interacts for this function with Aurora A Kinase (AURKA) [252], which has been already demonstrated to be deregulated by MINCR silencing in BL-2 cells [198]. In support of the emerging mechanisms between MINCR overexpression and silencing, we analyzed DEGs involvement in cancer and neurodegenerative disorders. Even if in SH-MINCR+ are present DEGs that participate in neurodegeneration and in SH-siMINCR DEGs involved in cancer, a clear trend emerged from the analysis showing a great role for MINCR overexpression in cancer and MINCR down-regulation in neurodegeneration. Some DEGs contribute to both the pathological conditions in different ways according to their expression levels. For example, in SH-siMINCR we found PACS1 upregulated that promotes mitochondrial cell death regulating BAX/BAK oligomerization. Mutations in PACS1 have been described in ALS patients [253]. Moreover, PACS1 participates also to tumour where, on the contrary, its down-regulation promotes gastric cancer [254]. Another example is WDR82 that we found down-regulated in SH-siMINCR. WDR82 is a component of the SET1A/SET1B histone H3-Lys4 methyltransferase complexes and it has been found down-regulated in ALS Drosophila model [255]. WDR82 is also upregulated in murine squamous cell carcinoma where it promotes the activity of pro-inflammatory genes [256]. The findings here reported show that MINCR has more than one mechanism of action depending on its expression level and suggest that it can participate to the pathogenesis of different diseases, not limiting its action to cancer. Indeed, these data support the idea that cancer and neurodegeneration can share key genes and pathways conversely regulated.

Finally, we investigated the effect of MINCR in ALS using differentiated SH-SY5Y cells as neuronal model. Through RNA pull down experiment we demonstrated that MINCR physically interact with Histone H1. Histone H1 family members are key component of chromatin and bind to the nucleosomal core particle around the DNA entry and exit sites. Their functions include, among the others, heterochromatin formation, specific gene expression and DNA repair [257]. It has been demonstrated that Histone H1 can be target of lncRNA resulting in remodeled chromatin structures and altered gene expression [258]. This finding, that still needs to be confirmed and investigated, could suggest the potential role of MINCR and its mechanisms of action.

Moreover, knocking-out MINCR through CRISPR-Cas9 system in differentiated SH-SY5Y cells, we demonstrated that KO cells showed hallmarks of apoptosis (cell

shrinkage, membrane bleb formation, detachment from the surface and aggregation), confirmed with Annexin V and 7-AAD staining, which showed that MINCR KO leads to increase in apoptosis, especially in late apoptosis. These data are potentially interesting since they suggest that MINCR down-regulation can promote cellular apoptosis. Moreover, alteration in cytoskeleton, especially in Beta-Tubulin III and Nestin, which are also neuronal marker, were detected in SH-KO after differentiation protocol, showing an impairment in axonal outgrowth.

In conclusion, dysregulation of RNA metabolism appeared to be a central mechanism in sALS. Our data brought the light on transcription activity alteration of MYC, paving a new way for future studies in MYC related pathways and network. Moreover, we highlighted also lncRNA alteration in sALS, especially demonstrating the mechanisms of action and targets of MINCR that, thus, can be considered a new possible interesting therapeutic target.

8. REFERENCES

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