Department of

Medicine and Surgery

PhD program in **Neuroscience**
Cycle **XXXIV** Curriculum in **Experimental Neuroscience**

Revealing the involvement of MALAT1, NEAT1, HOTTIP lncRNAs in Amyotrophic Lateral Sclerosis (ALS) via an induced pluripotent stem cell (iPSC)-derived muscle cell model

Surname **Giagnorio** Name **Eleonora**

Registration number **766021**

Tutor **Renato Emilio Mantegazza**

Supervisor **Stefania Marcuzzo**

Coordinator: **Maria Rosa Moresco**

ACADEMIC YEAR 2020/2021

Index

Abstract

Amyotrophic Lateral Sclerosis (ALS) is a neurodegenerative and fatal disease characterized by progressive cortical, bulbar and spinal motor neuron (MN) degeneration, leading to progressive muscle weakness, atrophy, paralysis and, ultimately, death. ALS can occur in two different forms: sporadic ALS (sALS) in ~90% of individuals and familial ALS (fALS). Different genes have been associated with fALS and/or sALS; C9ORF72–SMCR8 complex subunit (*C9ORF72*) is the gene most commonly linked to inherited ALS, followed by TAR DNA-binding protein 43 (*TARDBP*), superoxide dismutase 1 (*SOD1*) and FUS RNA-binding protein (*FUS*). Such genes affect several cellular functions, including oxidative stress (*SOD1*), RNA metabolism (*C9ORF72*, *TARBDP* and *FUS*), cytoskeletal organization [e.g. tubulin alpha-4a (*TUBA4A*) and profilin 1 (*PFN1*)] and autophagy [e.g. TANK-binding kinase 1 (*TBK1*) and optineurin (*OPTN*). ALS-associated mutant genes are ubiquitously expressed, thus alterations in structure, metabolism and physiology occur in different cell types, synergistically contributing to ALS degenerative pathways. It is generally accepted that ALS is primarily caused by MN death. However, growing evidence has shown that muscle is active and plays a crucial role in the disease onset and progression. Currently, there are no effective treatments for ALS. Indeed, one of the major aims in ALS research is the development of successful therapies, by deepening the knowledge of the molecular events leading to the degeneration of both MNs and muscle tissue.

It has become increasingly clear that RNA dysregulation is a key contributor to ALS pathogenesis. Among non-coding RNAs, long non-coding RNA (lncRNAs) are emerging as molecular contributors to ALS pathophysiology because of their role in regulating gene expression. LncRNAs, that are 300 to thousands nucleotides long, being more similar to mRNA than microRNAs, are key MN and muscle gene expression regulators. However, the exact contribution to ALS pathogenesis is still unknown.

Here, we analysed the expression levels of MALAT1, NEAT1 and HOTTIP lncRNAs, known to be involved in the development and homeostasis of the skeletal muscle, in a human induced pluripotent stem cell (hiPSC) model differentiated towards a myogenic destiny through a small molecule-based protocol, obtained from ALS patients and healthy controls.

The expression of key markers of skeletal muscle development was assessed by qPCR. Further, mRNA targets of the lncRNAs were predicted *in silico*, and validated by qPCR. We reported a differential lncRNA and mRNA target expression pattern in ALS-mutant cultures compared to controls, particularly at the mesodermal progenitor, early myocyte and myotube stages. Specifically, through hierarchical clustering analysis we identified specific clusters of lncRNA/target gene defining ALS cell lines, suggesting that an altered expression of these molecules might contribute to the disease pathogenesis.

Our findings on dysregulation of MALAT1, NEAT1, HOTTIP and their target genes in the iPSC-based ALS in vitro model provide new insights into ALS molecular basis, pointing out the possibility that altered muscle differentiation processes, depending on these lncRNAs, could eventually lead to an alteration in the architecture and mass of the skeletal and in its function, throughtout the disease progression. Further studies in genetically defined, or not defined, ALS patients, and

in other motor neuron diseases (MNDs), could help to deeply understand the synergistic effect of MALAT1, NEAT1 and HOTTIP in disease onset and/or progression, towards future development of patient-specific lncRNA-based therapeutic strategies for ALS and other MNDs.

1 Introduction

1.1. Amyotrophic Lateral Sclerosis

Amyotrophic Lateral Sclerosis (ALS) is a neurodegenerative disease characterized by progressive and selective cortical, bulbar and spinal motor neuron (MN) loss. ALS symptoms begin with focal weakness in the limbs, and spread inexorably to the other skeletal muscles, leading to progressively muscle wasting, atrophy, paralysis and ultimately death. The initial presentations of ALS vary according to disease onset. Some of them present a spinal-onset, but others about 30% show a bulbar-onset disease characterized by dysarthria and dysphagia [Hardiman O. et al, 2017].

However, a spectrum of non-motor manifestations is also common, since about 30%-50% of patients develop cognitive and/or behavioural impairment, whereas 13% of ALS patients show concomitant behavioural variant of frontotemporal dementia (FTD), from the onset [Huynh W. et al, 2020]. Average age of onset is 58–60 years with median survival from onset to death within two to four years, mainly due to respiratory failure [Hardiman O. et al, 2017].

Disease progression and survival vary among different disease subtypes, according to clinical features, with older age and bulbar onset having a worse outcome [Chiò A. et al, 2009]. Almost ∼90% of ALS cases are sporadic (sALS), whereas 10% are familial (fALS), showing different patterns of inheritance, including autosomal dominant,

autosomal recessive, X-linked or with no discernible family history [Boylan K. 2015].

As shown in Figure 1., many causative genes are associated with ALS, as C9orf72–SMCR8 complex subunit (*C9ORF72*), TAR DNA-binding protein 43 (*TARDBP*), superoxide dismutase 1 (SOD1) and FUS RNA-binding protein (*FUS*). These genes affect different cellular functions, mainly involving oxidative stress (*SOD1*), RNA metabolism (*C9ORF72*, *TARBDP* and *FUS*), cytoskeletal organization [e.g. tubulin alpha-4a (*TUBA4A*) and profilin 1 (*PFN1*)] and autophagy [e.g. TANK-binding kinase 1 (*TBK1*) and optineurin (*OPTN*)] [Hardiman O. et al, 2017]. The genetic heterogeneity reflects the different initial clinical presentations, making ALS struggling and lengthy to diagnose.

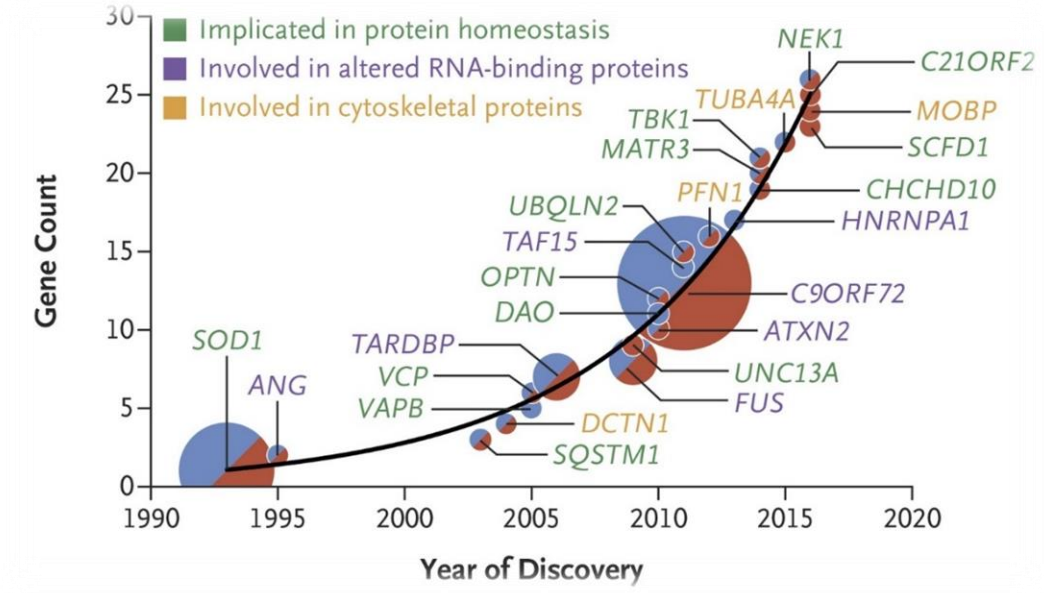

Figure 1. Timeline of gene discovery and pathogenic mechanism in ALS. Blue circles indicate genes associated with fALS, red circles indicate genes associated with sALS. The size of each circle reflects the proportion of all familial ALS cases associated with that gene. From Brown R. H. et al, 2017.

A common pathogenic hallmark of ALS is the aggregation and accumulation of protein inclusions in MNs. Pathological aggregations of

TDP43 are seen in up to 97% of ALS patients, although mutations in *TARDBP* are a rare cause of the disorder. In specific subtypes of ALS, other types of protein aggregates were observed, such as FUS, Sequestosome-1 (p62), OPTN and Ubiquilin 2 (UBQLN2) inclusions [Blokhuis A.M. et al, 2013].

It has become increasingly clear that RNA dysregulation is a key contributor to ALS pathogenesis, especially in *C9ORF72*, *TARBDP* and *FUS* mutant form of ALS.

The GGGGCC hexanucleotide repeat expansion in the first intron or promoter region of *C9ORF72* gene leads to the accumulation of repeat-containing RNA foci in ALS patient tissues [Gendron T.F. et al, 2013]. In turn, RNA foci lead to RNA-binding protein mislocalization causing RNA metabolism alterations [Donnelly C. J. et al, 2013]. It is still not clear how *C9ORF72* expansion causes ALS for toxic gain of function, loss of function, or both.

TARDBP gene codes for TDP43 protein that has a major role in different stages of mRNA processing, as splicing, stability and transport [Buratti E. and Baralle F. E. 2008]. TDP43 is normally nuclear, but in case of *TARDBP* mutations, it is mislocalized to the cytoplasm and forms aggregates. TDP43 aggregation provokes a loss of function of TDP43 and thus a dysregulated RNA metabolism and a gain of function, since the aggregates are cytotoxic [Kabashi E. et al, 2010].

FUS plays multiple roles in RNA processing by directly binding to RNA, with an essential role in splicing regulation [Lagier-Tourenne C. et al, 2012]. Indeed, it was shown that loss of function of *FUS* led to splicing changes in more than in more than 300 genes in mice brain [Lagier-Tourenne C. et al, 2012].

Transgenic *SOD1* mouse is the most known and used ALS animal model of the disease [Gurney M.E. et al, 1994]. However, the broad

variety of genetic mutations identified in ALS led to generate several transgenic ALS animal models of different species as *Caenorhabditis elegans*, *Drosophila melanogaster*, *Danio rerio*, *Rattus norvegicus* and *Sus scrofa* in which were introduced different mutations as *TARDBP*, *FUS*, *C9ORF72*, *VAPB* and *VCP* [Picher-Martel V. et al, 2016]. These models mainly recapitulate the molecular and cellular features that mimic those characteristic of fALS and sALS.

The discovery of human induced pluripotent stem cells (iPSCs) revolutionized the approach to human disease modelling [Takahashi K. et al, 2007]. IPSCs are a unique tool for studying the cellular and molecular mechanisms of neurodegenerative diseases including ALS, since these cells are patient-specific *in vitro* model and they are able to differentiate in different cell types [Fujimori K. et al, 2018]. Moreover, considering the great genotypic and phenotypic heterogeneity in ALS, a personalized model better recapitulates the epidemiology of the disease and allows the progress of precision medicine [Hardiman O. et al, 2017].

Treatment of ALS is managed by a multidisciplinary research approach, but only two compounds, Riluzole and Edaravone, were approved by the Food and Drug Administration (FDA). Riluzole is a glutamate antagonist, acting as inhibitor of the voltage-gated sodium channels on presynaptic neurons, reducing the excessive glutamatergic neurotransmission; Edaravone is a free radical scavenger, protecting cells against oxidative stress. However, Riluzole and Edaravone show modest and few benefits on survival [Hardiman O. et al, 2017].

Recently, a multicentric phase 2 clinical trial demonstrated that Guanabenz, a drug targeting the unfolded protein response, significantly slowed the progression of bulbar ALS patients, even though the mechanisms underlying the effect on such disease subtype

are unknown [Dalla Bella E. et al, 2021].

1.2. Skeletal muscle as a key site of ALS pathophysiology

In ALS, the major intrinsic pathogenic mechanisms occurring within MNs include massive excitotoxic insult, axonal transport deficiency and neurofilament aggregation [Tsitkanou S. et al, 2019; Le Gall L. et al, 2020]. However, a specific MN vulnerability arises from the combination of several factors and events that result from MN interaction with surrounding non-neuronal cells, including protein misfolding and aggregation, mitochondrial alterations, oxidative damage, insufficient growth factor signalling, inflammation and glutamatergic excitotoxicity [Ross C. A. and Poirier M. A. 2004; Raimondi A. et al, 2006; Said A. M. et al, 2000; Ragagnin A. M. G. et al, 2019; Menon P. et al, 2015].

ALS is considered a 'multi-systemic' disease, where MN damage is enhanced by impaired neighbouring cells, such as activated astrocytes and microglia, which contribute to generate an inflammatory state [Lobsiger C. S. and Cleveland D. W. 2007]. It is generally accepted that ALS is primarily caused by MN death. However, growing evidence has shown that muscle is active and crucial role in the onset and progression of ALS [Scaricamazza S. et al, 2021].

1.2.1 The skeletal muscle

Skeletal muscle is one of the most dynamic and plastic tissues of the human body. It is composed by a heterogeneity of cell types as myofibers, fibroblasts, endothelial cells, blood cells, neurons and others. Myofibers are the basic unit of the skeletal muscle and are multinucleated since they are generated by the fusion of multiple myotubes [Cretoiu D. et al, 2018].

Of note, the ability to adapt to physiological and ectopic stimuli, as physical exercise or disease state, is a distinctive and unique characteristic of the skeletal muscle. The muscle plasticity is a useful characteristic for responding to any circumstance with the best performance, also in case of disease. This feature slows down disease progression and counteract myodegeneration. In particular, muscles regenerate or modify the composition of their fibre phenotypes, switching from fast to slow-twitch fibres or vice versa, or they change their cross-sectional area and thus the amount of force they can produce [Hoppeler H. 2016].

1.2.2. Skeletal muscle regeneration

The skeletal muscle has the ability to self-regenerate by the proliferation and differentiation of satellite cells (SCs). SCs mostly originate from Paired box (PAX) 3^+ and $PAX7^+$ embryonic progenitor cells. Under basal conditions, SCs are quiescent and reside in a muscle niche located between the sarcolemma and basal lamina. During muscle damage and denervation or in exercise condition, SCs proliferate and differentiate into mature myofibers to counteract the damage or to positively support the physical activity.

The quiescence state is maintained through the expression of *PAX3* and *PAX7* genes, wheras SC activation is determined by a down regulation of *PAX3* and *PAX7* genes and an up regulation of Myoblast determination protein (*MYOD*) and Myogenic factor 5 (*MYF5*) genes. At this stage, SCs differentiate into myoblasts, myogenic precursors characterised by an intense proliferative capacity (Figure 2.A.).

Interesting, the subsequent activation of Myogenin (MYOG), Myocyte enhancer factor 2C (MEF2C) and MYF6, determines the start of terminal differentiation step characterised by the fusion of several myoblasts to generate multinucleated myofibers (Figure 2.A.).

The morphological unit of the mature skeletal muscle is the myofiber positive for Myosin heavy chain (MHC) that is the terminal differentiation marker of the myogenic process [Chal J. and Pourquié O. 2017].

1.2.3. Fibre type switch

In mammalian skeletal muscles, two types of myofibres exist and are classified as 'slow-twitch' (type I) or 'fast-twitch' (type II) fibres.

Slow-twitch fibres express myosin heavy chain (MHC)-IB (encoded by *MYH7*) and are characterized by a slow contraction capacity and a slow rate of fatigue; they are innervated by small-calibre axons, have a small diameter, high myoglobin content, numerous capillaries, oxidative metabolism and a high number of mitochondria. Because of these characteristics, slow-twitch fibres are involved in sustained, tonic contractile events [Schiaffino S. and Reggiani C. 2011].

In contrast, fast-twitch myofibres contract rapidly and are susceptible to fatigue. Fast-twitch myofibres express MHC-IIA (encoded by *MYH2*), MHC-IIX (MYH1) and MHC-IIB (MYH4); they are characterized by a low number of mitochondria and are innervated by large-calibre axons. Fast IIA-twitch fibres employ an oxidative metabolism and are responsible for a fast contraction time with a high resistance to fatigue. In turn, IIB fibres have the biggest diameter and the lowest amount of mitochondria, they rely on a glycolytic metabolic pathway, which guarantee a very quick contraction but a high susceptibility to fatigue.

Skeletal muscle fibres are generally intermingled within a single muscle group and different muscle groups have varying proportions of fibre types [Schiaffino S. and Reggiani C. 2011].

Of note, myofibres change from fast-twitch to slow-twitch type to allow adaptation to different events or stimuli, a phenomenon called fibre-type switching, as shown in Figure 2.B.

Fibre-type switching is observed as a consequence of exercise and enhances muscle fatigue resistance. It is also a preventive mechanism to counteract myodegeneration, as observed in ALS patients and mice [Peggion C. et al, 2017; Telerman-Toppet N. and Coërs C. 1978].

1.2.4. Regulation of muscle mass

Skeletal muscle fibres have the ability to change their cross-sectional area and thus the amount of force they can produce. Muscle fibre size is influenced by a wide range of physiological inputs as physical exercise, nutrients, growth hormones, cytokines, and other secreted factors. Skeletal muscle tissue grow by two mechanisms: hyperplasia (cell number increase) and hypertrophy (cell size increase) [Schiaffino S. et al, 2013].

The postnatal growth of skeletal muscles is primarily due to hypertrophy, which is due to an increasing net protein content, that causes an elongation and expansion of the diameter of myofibers [Li M. et al, 2015].

The control of muscle mass is achieved through the regulation of the balance between protein synthesis and degradation in skeletal muscle fibres. This balance is regulated by Protein kinase B (*AKT*) pathway, via two major branches: the AKT/ mammalian target of rapamycin (*mTOR*) pathway that controls protein synthesis and the AKT/ Forkhead box O (*FOXO*) one controlling protein degradation. These pathways are activated respectively by Insulin-like growth factor 1 (*IGF1*), acting as a positive regulator of muscle growth, and by

Myostatin (*MSTN*), which negatively regulates muscle mass (Figure 2.C.) [Francaux M. and Deldicque L. 2019].

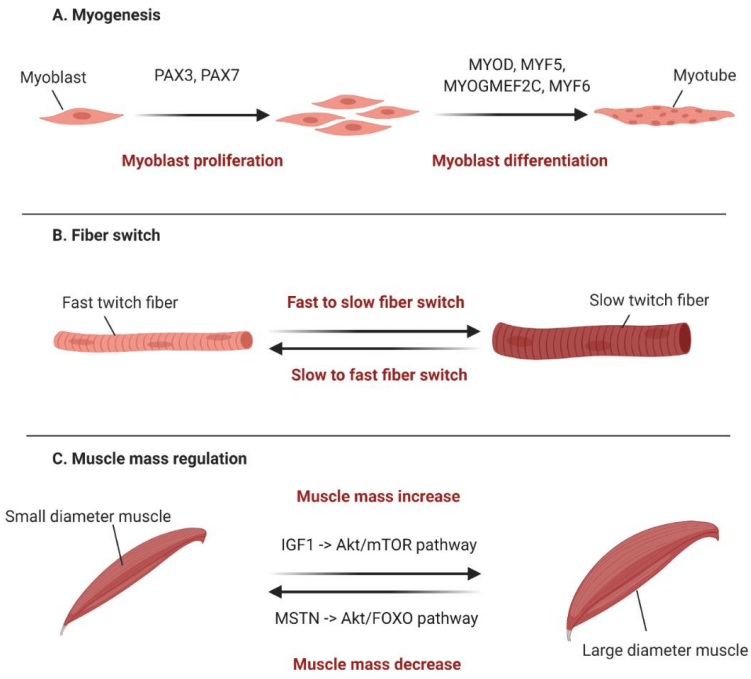

Figure 2. Overview of skeletal muscle plastic functions. A. myogenesis, B. fibre switch and C. mass regulation.

Muscle mass increases following progressive exercise training and in contrast, it is lost in case of neuromuscular disease, chronic disease, ageing, as well as limb immobilization or prolonged bed rest due to injury or trauma.

Skeletal muscle atrophy is a well-known ALS symptom and different studies investigated the molecular pathways causing it [Halon-Golabek M. et al, 2018].

1.2.5. Skeletal muscle in ALS

The general assumption that ALS is only due to MN degeneration, is widely debated. Different studies support the idea that ALS is caused by a cell-autonomous dysfunction occurring in the MNs, suggesting that ALS related mutant genes only affect MNs and thus that the neuronal damage is the unique determinant of disease onset and progression. The selective expression of G93A-*SOD1* mutant protein in the neurons of adult mice, was shown to be sufficient to induce the disease in transgenic mice [Jaarsma D. et al, 2008]. Indeed, according to the so-called "dying forward" theory, MN dysfunctions begin within the cell body of upper MNs, at the cortical level. Cortical MN hyper-excitability causes the degeneration of spinal MNs, via an anterograde trans-synaptic glutamatergic mechanism, since axonal electrophysiological dysfunctions or axonal transport defects were observed in ALS [Menon P. et al, 2015; Marcuzzo S. et al, 2019]. However, all ALS causative genes are ubiquitously expressed, thus non-neuronal cells may contribute to MN loss, then resulting in the disease.

Indeed, previous studies demonstrated the contribution to ALS of many different cell types, as microglia, astrocytes, oligodendrocytes, T cells and myofibers [Ilieva H. et al, 2009]. These studies support the so-called "dying back" hypothesis, which postulates that MN dysfunctions begin distally, at the neuromuscular junction (NMJ) level or in the peripheral tissues and progress toward the cell body. For this reason, ALS was also described as a distal axonopathy, which, via a retrograde signalling cascade, leads to MN death [Moloney E.B. et al, 2014; Rocha M.C. et al, 2013].

Growing evidence define ALS as multi systemic disease, since it arises within different cell types, all contributing to a toxic environment, which sustain MN intrinsic degeneration. Based on these evidences, the

skeletal muscle has a relevant role in ALS pathogenesis [Ilieva H. et al, 2009].

In G93A-*SOD1* ALS mouse model, it was showed an early reduction of the skeletal muscle volume before any clinical sign and neurodegeneration in the motor nuclei [Marcuzzo S. et al, 2011]. Mutant G93A-*SOD1* or G37R-*SOD1* proteins, expressed exclusively in murine muscles, lead to progressive muscle inactivity and paresis caused by a continuous myofiber loss. These conditions are accompanied by NMJ and spinal cord MN alterations, suggesting that the presence of mutant *SOD1* in the muscle is sufficient for MN degeneration in mice [Martin L.J. and Wong M. 2020].

As shown in Figure 3., several cellular properties and functions are dysregulated in ALS myofibers, including alterations in the structure, DNA integrity, dynamics and function of mitochondria. Mitochondrial changes alter cellular respiration rate and ATP production, leading to calcium homeostasis loss, stimulation of pro-apoptotic signalling and increased ROS levels [Smith E.F. et al, 2019]. Mitochondrial dysfunctions and the accumulation of reactive oxygen species (ROS), that are partially causative for the hypermetabolic state of ALS, have been observed in human muscle biopsies during disease progression [Echaniz-Laguna A. et al, 2006]. Another ALS pathophysiological alteration that is already well-known in ALS MNs is an imbalanced proteostasis, in particular, the cytoprotective autophagic pathways are suppressed in ALS skeletal muscle, whereas apoptosis is enhanced, leading to a booster in muscle atrophy and ALS progression [Xiao Y. et al, 2015]. In turn, these alterations actively contribute to the accumulation of protein aggregates, consisting of misfolded proteins, mainly TDP43, in the muscle tissue, which are associated with myogenic muscle degeneration [Mori F. et al, 2019]. Moreover, satellite cells (SCs) from ALS muscle, fundamental for myofiber regeneration,

exhibit a high proliferative potential but display an abnormal senescent-like morphology, characterized by an elevated number of vacuoles, and altered capacity to differentiate into myotubes, contributing to muscle defects [Scaramozza A. et al, 2014].

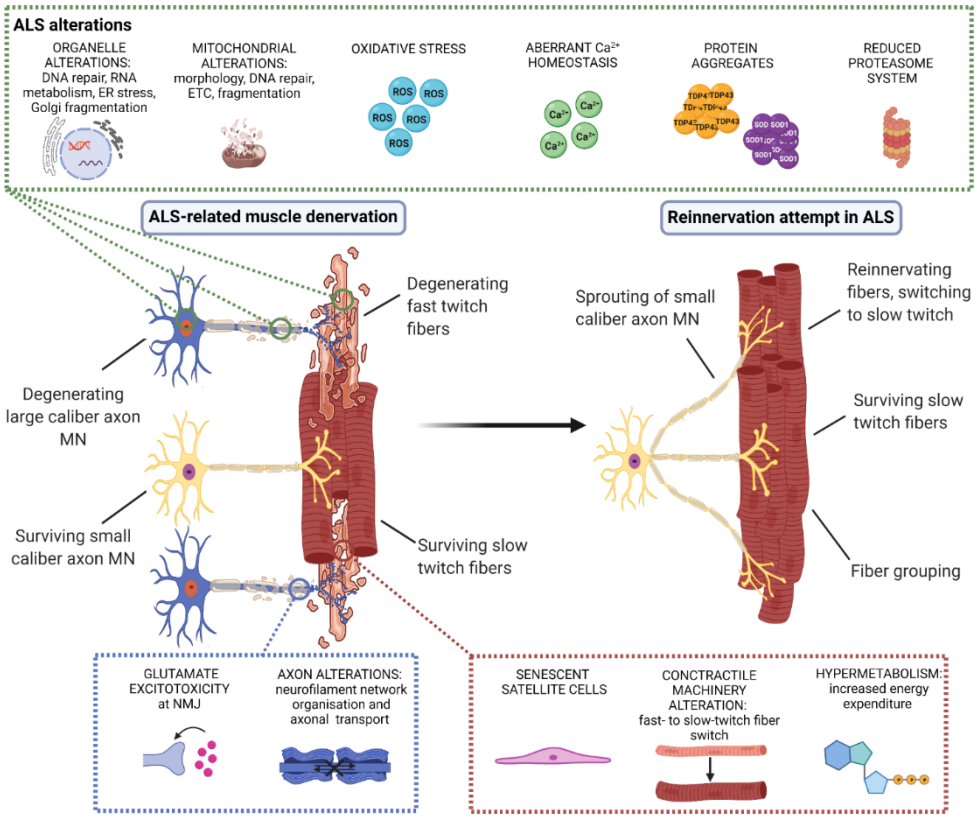

Figure 3. Fast-to-slow myofibre type conversion in the context of ALS pathogenesis. Organelle and mitochondrial alterations, oxidative stress, aberrant Ca2+ homeostasis, protein aggregates and reduced proteasome activity are all pathogenic events implicated in MN degeneration and muscle atrophy in ALS (see inset in green). Bulbar and spinal cord MNs, connecting upper MNs to skeletal muscle, are affected by glutamatergic excitotoxicity and axon alterations that contribute to degenerative events (see inset in blue). Senescent satellite cells and hypermetabolism are a known characteristic of ALS atrophic muscle (red inset). From Giagnorio E. et al, 2021

Fast-to-slow myofiber conversion acts as a mechanism to counteract ALS myodegeneration [Hegedus J. et al, 2008]. Specifically, in ALS it was observed an early degeneration of large-calibre axon MNs, which in turn is accompanied by degeneration of fast-twitch muscle fibres. Sprouting of neighbouring intact small calibre axons determines the innervation of multiple fibres by the same motor axon, leading to fibre clustering and promoting reinnervation. As large-diameter axons degenerate early in ALS, reinnervation becomes dependent on small-diameter axons, which then lead to muscle fibre grouping, thereby causing their fast-to-slow phenotypic change and hence a slower muscle-contraction capacity (Figure 3.) [Giagnorio E. et al, 2021].

The involvement and accessibility of the skeletal muscle in ALS suggests that it could represent a crucial and accessible site for curative interventions, particularly for gene and cellular therapies. Intramuscular injections of lentiviral vectors specifically inhibiting *SOD1* expression in ALS G93A-*SOD1* mice result in improved survival of vulnerable brainstem and spinal cord MNs [Ralph G.S. et al, 2005]. Furthermore, intramuscular injections of neuregulin 1 type I (NRG1-I), a trophic factor highly expressed in normal MNs and NMJs and implicated in muscle reinnervation processes, shows positive effects on survival of G93A-*SOD1* mice [Mòdol-Caballero G. et al, 2020]. Interestingly, repeated intramuscular transplantation of human umbilical cord blood-derived mesenchymal stem cells into the gastrocnemius of G93A-*SOD1* mice significantly improves motor activity and prolonged survival [Kook M.G. et al, 2020]. In ALS patients, intramuscular injections of autologous mesenchymal stem cells secreting neurotrophic factor (NTF) have been used in a phase 1/2 and 2a clinical trial. The treatment was safe and well tolerated (primary outcome) over the study follow-up period and leads to a reduction of the disease progression rate (secondary outcome), accompanied by a lower muscle volume decline [Petrou P. et al, 2016]. These studies

indicate that skeletal muscle is a promising therapeutic target. Therefore, treatment strategies aimed at improving muscle tissue homeostasis and function could lead to a general disease improvement due to the strict 'MN–muscle' communication. Nevertheless, the development of such types of strategies would greatly benefit from a deeper understanding of the molecular alterations in ALS.

Intrinsic skeletal muscle deterioration plays a crucial role in the disease and contributes to ALS progression. Currently, there are no effective treatments for ALS, highlighting the need to obtain a deeper understanding of the molecular events underlying degeneration of both MNs and muscle tissue, with the aim of developing successful therapies.

1.3. Long non coding RNAs

Genomes are extensively transcribed and give rise to thousands of long non-coding RNAs (lncRNAs), which are defined as RNAs longer than 200 nucleotides that are not translated into functional proteins. The number of functional lncRNAs is still debated, since the number of known protein-coding genes has remained stable over recent years whereas the number of known lncRNAs is still increasing [Mercer T.R. and Mattick J.S. et al, 2013]. They are widely expressed and have key roles in gene regulation.

Several studies have begun to unravel how the biogenesis of lncRNAs is distinct from that of mRNAs and it is associated with their specific subcellular localization and functions [Statello L. et al, 2020].

Based on their localization and interactions with DNA, RNA and proteins, lncRNAs modulate chromatin structure and function, regulate the assembly of membraneless nuclear bodies, alter the stability of cytoplasmic mRNAs and influence different signalling pathways. These functions ultimately affect gene expression in different biological and

pathophysiological contexts, such as neurological disorders. Tissue-specific and condition-specific expression patterns suggest that lncRNAs are potential biomarkers and new therapeutic targets for the treatment of different diseases [Statello L. et al, 2020].

1.3.1. Biogenesis and classification

Most lncRNAs are generated via pathways similar to that of protein-coding genes, since they are transcribed by RNA polymerase II, they undergo a splicing phase and have 5'-end m7 G caps and 3'-end poly (A) tails.

LncRNA genes are less evolutionarily conserved, because they contain few exons that are less abundantly expressed than mRNAs but they show a very high tissue-specific expression, as lncRNAs muscle specific, including linc-MD1 and lncMyoD [Statello L. et al, 2020; Legnini I. et al, 2014; Gong C. et al, 2015]. LncRNAs loci are often in strong association with protein coding genes, but they are transcribed independently, even though it was shown that small polypeptides (micropeptides) origin from lncRNAs [Anderson D.M. et al, 2015]. According to their localisation in the genome, lncRNAs are classified as intergenic lncRNA (lincRNAs) if the lncRNA does not overlap with a protein-coding transcript, or as genic lncRNAs if lncRNAs overlap with a protein-coding transcript [Ransohoff J.D. et al, 2018]. Genic lncRNAs are further classified as a) Exonic lncRNAs that overlap with exonic regions of a protein-coding gene; b) Intronic lncRNAs that merge with intronic regions of a protein-coding gene; c) Overlapping lncRNAs that overlap with a protein-coding gene in the sense or antisense direction. However, lncRNA classification is confusing and overlapping, since several transcriptome sequencing studies are accumulating, generating ambiguity and lack of clarity in the field [revised in St Laurent G. et al, 2015].

1.3.2. Structure

LncRNAs have heterogenic structures, compared to microRNAs (miRNAs), reflecting their multifaceted functional properties. LncRNAs are characterized by a modular architecture that adds a further level of complexity to non protein-coding RNAs (ncRNAs) activity [Guttman M. et al, 2012].

Each module corresponds to an RNA functional domain, including tens to hundreds of nucleotides, characterised by an independently-folding secondary structure which confers to the domains an independent function [Guttman M. et al, 2012].

Clearly, lncRNA secondary structures reflects the secondary structure motif of their each target [Mathews D.H. et al, 2010]. The secondary structures independently fold and subsequent tertiary interactions occur, resulting in the hierarchical assembly of RNA structure. Interestingly, lncRNAs tend to acquire complex secondary and tertiary structures and their function is dependent on structural conservation rather than primary sequence conservation.

Another characteristic of lncRNA structure is the flexibility. Indeed, junctions confer rigidity the molecule wheras internal loops are critical to modulate lncRNA flexibility of the helical stems [Zhao C. et al, 2015].

1.3.3. Function

Growing evidence highlights the role of lncRNAs as positive or negative regulators of gene expression at the epigenetic, transcriptional, or post-transcriptional level and they are responsible for the integrity of nuclear structures [Laneve P. et al, 2021].

Compared with mRNAs, several lncRNAs are retained in the nucleus and, thanks to their long sequences, they contain multiple functional

domains that permit to interact with different molecules as DNA, mRNAs, miRNAs and proteins, at the same time coordinating their activity in time and space [Marchese F.P. et al, 2017]. Interestingly, lncRNAs regulate the expression of either nearby genes acting in cis in the nucleus or genes elsewhere in cells acting in trans in the nucleus via chromosomal looping or cytoplasm [Marchese F.P. et al, 2017].

LncRNAs positively or negatively modulate gene expression at different levels, via their interaction with DNA, mRNAs, miRNAs and proteins, as shown in Figure 4..

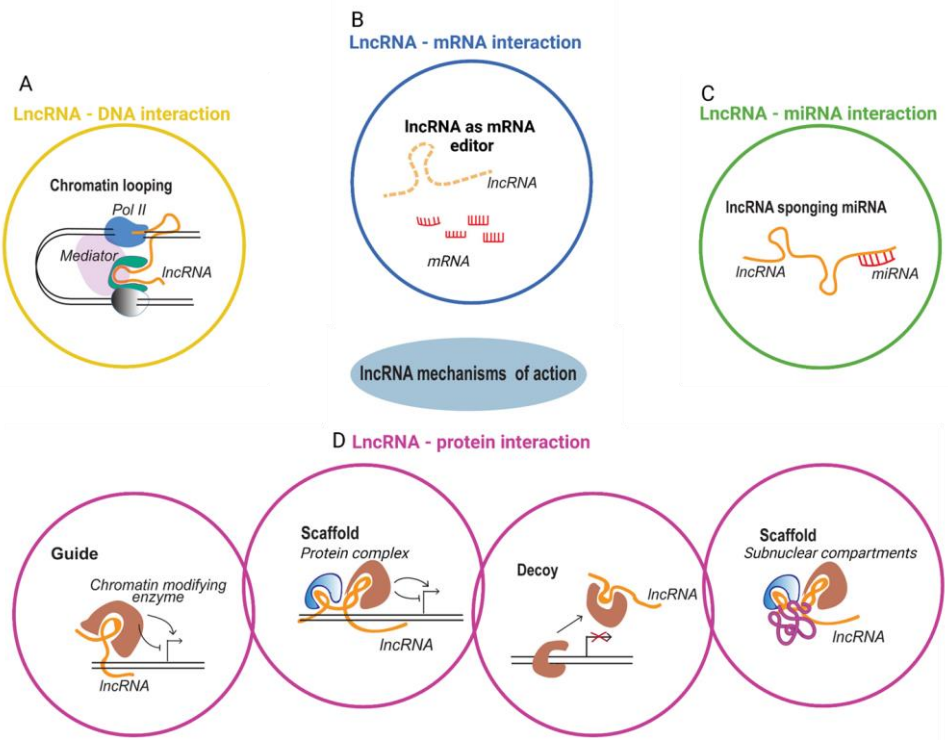

Figure 4. LncRNA mechanisms of action. A describes lncRNA-DNA interaction; B lncRNA-mRNA interaction; C lncRNA-miRNA interaction and D lncRNA-protein interaction. Adapted from Sweta S. et al, 2019.

- **DNA interaction**. LncRNAs modulate chromatin structure and functionality via three mechanisms.

- LncRNAs directly interact with the DNA, in a sequence-specific manner, via the formation of a **triple helix**. The chromatin looping of two distant regions of the genome, as enhancer and promoter, allows to bring these two regions in close proximity thus to modulate target-gene transcription. Interestingly, it was shown that MEG3 causes chromatin looping of promoter-distal regulatory regions of Transforming growth factor beta 1 (*TGFβ*) genes modulating the activity of TGFB pathway genes [Mondal T. et al, 2015].
- LncRNAs act as **scaffolds** for chromatin modifiers or transcription factors. By protein-RNA photo-crosslinking and mass spectrometry experiments on embryonic stem cell nuclei, many RNA-binding regions were identified in several chromatin regulators, as Ten-eleven translocation 2 (TET2), demonstrating their ability to bind RNA molecules [He C. et al, 2016]. Indeed, TET2-interacting lncRNA (TETILA) guides the position of TET2 on the genome in turn activating target gene expression [Zhou L. et al, 2019].
- LncRNAs combine these two functions firstly forming a DNA-RNA **triplex helix** that is subsequently recognised and act as **scaffold** for chromatin-modifying enzymes. Of note, Khps1 is transcribed in antisense orientation to the proto-oncogene Sphingosine kinase 1 (SPHK1) directly associates with the promoter of *SPHK1* gene by DNA-RNA triplex. This mecchanism leads to the anchoring of effector proteins to the gene promoter stimulating the expression of the proto-oncogene [Postepska-Igielska A. et al. 2015].

- **mRNA interaction.** Through the formation of RNA-RNA duplex by direct base pairing, lncRNAs alter the transcriptome and proteome by affecting mRNA splicing and editing. Specifically, lncRNA-pre-mRNA duplex conformation causes the blockade of spliceosome

assembly impairing intron-exon junction [Beltran M. et al, 2008]. Indeed, Snail1 lncRNA alters Zeb2 mRNA processing by masking an internal ribosome entry site. At this site, Snail1 prevents the binding of the spliceosome to this specific splice region causing the retaining of an idditional intron that is fundamental for the correct translation of Zeb2 [Beltran M. et al, 2008]. Moreover, lncRNA-mRNA duplex is a good substrate for RNA adenosine deaminase, an enzyme that converts adenine to inosine, altering the base pairing. Several A to I editing sites are contained in lncRNA sequences leading to a change in the lncRNA secondary structure and therefore in their functions [Salameh A. et al, 2015]. Prostate cancer antigen 3 (PCA3) lncRNA negatively targeting the tumor suppressor gene Prune homolog 2 with BCH domain (*PRUNE2*), thereby promoting malignant cell growth. PCA3 binds PRUNE2 mRNA generating a lncRNA-mRNA duplex which is recognised by adenosine deaminase acting on RNA (ADAR) proteins. These deaminases A to I edit PCA3 or PRUNE2 leading to a downregulation of the edited RNA [Salameh A. et al, 2015].

- **miRNA interaction.** According to the competing endogenous RNA (ceRNA) hypothesis, lncRNA-miRNA interaction impairs miRNA activity through their sequestration, acting as miRNA "sponges" or sequestrator [Salmena L. et al, 2011]. Interestingly to note that the lncRNA HULC sequesters the miR-372 modulating its transcriptional upregulation in hepatocellular carcinoma [Wang J. et al, 2010].
- **Protein interaction.** LncRNAs mainly exist in the form of protein complexes and act as guides, scaffolds and decoys for different proteins. Moreover, lncRNAs are structural components of subnuclear structures [Bond C.S. et al, 2009].
    - LncRNAs act as **guides**. A lncRNA identifies and binds a specific DNA loci acting as guide for recruiting the proteins and enzymes towards the chromatin sites to regulate

downstream gene expression. For example, lncRNAs guide the activity of polycomb repressive complex (PRC) 1 and 2 to target genes. PRC1 and PRC2 cause the trimethylation of histone H3 on lysine 27 (H3K27) leading to gene repression [Fiorenzano A. et al, 2019]. For example, X-inactive specific transcript (Xist), via this mechanism, is responsible for the entire inactivation of one X-chromosome in female somatic cells by forming a transcriptionally repressed heterochromatic structure [Cerase A. et al, 2014].

- LncRNAs act as **scaffolds** to create protein complexes in specific regions of the genome. They recruit chromatin remodelling complexes thus inducing a conformational changes in the state of the chromatin, causing a negative or positive regulation of the gene expression in specific loci. By this function, lncRNAs recruit, bind and complex PRC1, PRC2 and other proteins to inhibit gene expression in discrete regions of the genome [Tsai M.C. et al, 2010]. HOX transcript antisense RNA (HOTAIR) serves as a scaffold for PRC2 and LSD1/CoREST/REST repressor complex. The interaction between these factors leads to histone modifications, responsible for Homeobox D cluster (*HOXD*) silencing [Tsai M.C. et al, 2010].
- LncRNAs act as **decoys** to remove proteins from target loci thus positively or negatively regulating gene expression or alternative splicing. LncRNAs sequester splicing factors to modulate their nuclear distribution, therefore regulating the alternative splicing of pre-mRNAs at a precise time, place, and concentration [Tripathi V. et al, 2010].

- LncRNAs are structural scaffold for the formation and regulation of **subnuclear compartments** including speckles and paraspeckles as MALAT1 and NEAT1, respectively [Yang L. et

al, 2011]. Speckles and paraspeckles are ribonucleoprotein bodies, enriched in pre-mRNA and splicing factors, located in the interchromatin space of cell nuclei. These compartments play a relevant role in regulating the gene expression and RNA splicing [Mao Y.S. et al, 2011].

1.3.4. LncRNAs in the skeletal muscle

In view of the critical regulatory function of the RNA metabolism and the crucial role of muscle in ALS pathogenesis, muscle-specific lncRNAs are worthy of further investigation as potential molecular targets of therapeutic strategies to improve symptoms and counteract disease progression.

LncRNAs are effective regulators of muscle development, myogenesis and differentiation in both physiological and pathological conditions as described in Table 1. LncRNA yin yang T (yylncT) is involved in the development of the skeletal muscle during the first days of the embryo life. Indeed, it activates *BRACHYURY (T)* locus, leading to mesodermal cell fate transition during gastrulation [Frank S. et al, 2019]. Moreover, Maternally expressed gene 3 (Meg3) was shown to promote skeletal muscle development by interacting with PCR2 during embryogenesis, but it also has a role in the postnatal myogenesis, stimulating myoblast identity differentiation via regulating epithelial-mesenchymal transition by the repression of TGFβ signalling [Dill T.L. et al, 2021].

The function of the most lncRNAs was deepen in the adult myogenesis and differentiation processes because of their possible contribution to muscle regeneration. Several lncRNAs act as sponges over miRNAs, as lncMyoD, Long intergenic non-protein coding RNA muscle differentiation 1 (lincMD1), Linc-smad7 and long non coding RNA Insulin receptor substrate 1 IRS1 (lncIRS1) over miR-370-3p, miR133/135, miR-125b and miR-15, respectively. Via the ceRNA

mechanism, these lncRNAs stimulate muscle differentiation [Zhang P. et al, 2021; Cesana M., et al, 2011; Legnini I. et al, 2014; Song C. et al, 2018; Li Z. et al, 2018]. Interestingly, lncMyoD is under the transcriptional control of *MYOD1* itself, suggesting a central regulatory role for the lncRNA in the myogenic differentiation process. Moreover, by binding and inhibiting IGF2-mRNA-binding protein 2 (*IMP2*), lncMyoD, blocks myoblast proliferation, in turn creating a permissive state for differentiation [Gong C. et al, 2015]. LncRNA muscle anabolic regulator 1 (MAR1) acts as a miR-487b sponge to regulate Wnt5a protein, resulting in promoting muscle differentiation and regeneration, whereas Myogenesis-associated lncRNA (lnc-mg) promotes myogenesis by functioning as a ceRNA for both miRNA-125b and miR-351-5p [Zhu M., et al, 2017; Du J., et al, 2019]. LncRNAs promote the myogenesis acting on the expression of myogenic regulatory factors (MRF), a transcription factors that controls the determination and differentiation of skeletal muscle cells during postnatal myogenesis, indirectly. For instance, Mechanical unloading-induced muscle atrophy-related lncRNA (lncMUMA) promotes myogenic differentiation by functioning as a miR-762 sponge in turn regulating the core myogenic regulator *MYOD* [Zhang Z.K. et al, 2018]. The lincMD1 and miR-133/135 pairing inhibits the expression of *MAML1* and *MEF2C* activating the muscle-specific gene expression [Cesana M., et al, 2011; Legnini I. et al, 2014]. Linc-YY1 is transcribed from the promoter of the transcription factor Yin Yang 1 (*YY1*) gene and interacts with YY1 to evict YY1/PRC2 activating the gene expression of myogenic factors in trans [Zhou L. et al, 2015]. However, lncRNAs are directly involved in the transcriptional control of MRFs, as Irm, which regulates the expression of myogenic gene *MEF2D*, promoting the assembly of MYOD1/MEF2D, thus regulating myogenesis [Sui Y., et al, 2019]. Linc-RNA activator of myogenesis (linc-RAM), Long non-coding steroid receptor RNA activator (SRA) and MYOD1 upstream noncoding

(MUNC) recruit and activate the *MYOD* gene promoting muscle growth and regeneration [Yu X. et al, 2017; Caretti G., et al, 2006; Mueller A., et al, 2015]. LncRNAs influence myoblast fusion at the end of myogenesis as lnc-mg. By inducing the expression of myomaker (*MYMK*), lnc-mg affects a muscle-specific transmembrane protein harbouring a critical role in the generation of multinucleated myotubes beyond its ceRNA activity [Militello, G., et al, 2018]. Moreover, Myogenin promoter-associated long non-coding RNA (Myoparr), a lncRNA derived from the upstream region of the *MYOG* gene, has a dual role in myogenesis: 1) it is involved in myoblast cell cycle withdrawal by the transactivation of myo-miRNAs expression; 2) it is responsible for the activation in cis of *MYOG* transcription through the interaction with DEAD-Box Helicase 17 (Ddx17)/P300/CBP-associated factor (PCAF) and *MYOG* promoter allowing the entrance of myoblasts into myogenic differentiation [Hitachi K., et al, 2019].

In addition, lncRNAs have a negative role in controlling myogenic differentiation processes, in turn supporting the proliferative myoblast state inhibiting their differentiation. Indeed, lncRNA silent information regulator 1 (Sirt1 AS) forms an RNA duplex to promote Sirt1 mRNA translation by competing with miR-34a inhibiting muscle formation. AK017368 lncRNA has the ability to induce proliferation and inhibit myoblast differentiation by sponging miR-30c and inducing fibre hypertrophy in mouse [Wang G.Q. et al, 2016; Liang T. et al, 2018]. Finally, SYNPO2 intron sense-overlapping lncRNA (SYISL) promotes proliferation and inhibits myoblast differentiation by silencing MYOG and myosin heavy chain (MHC) 4 genes [Jin J.J. et al, 2018].

lncRNA	Function	Target	Reference
AK017368	Myoblast proliferation	miR-30c	Liang T. et al, 2018

AtroInc-1	Muscle wasting	*ABIN-1*	Sun L. et al, 2018
Chronos	Atrophy	*EZH2*	Neppl R.L. et al, 2017
Irm	Myoblast differentiation	*MEF2D*	Sui Y., et al, 2019
LINC00961	Inhibits regeneration	v-ATPase	Matsumoto A. et al, 2016
lincMD1	Myoblast differentiation	miR-133/135	Legnini I. et al, 2014; Cesana M., et al, 2011
linc-RAM	Regeneration	*MYOD*	Yu X. et al, 2017
Linc-smad7	Myoblast differentiation	miR-125b	Song C. eta al, 2018
Linc-YY1	Myoblast differentiation	*YY1/PCR2*	Zhou L. et al, 2015
Lnc-31	Myoblast proliferation	*ROCK1*	Ballarino M. et al, 2018; Dimartino D. et al, 2018
lncIRS1	Myoblast differentiation	miR-15	Li Z. et al, 2018
lnc-mg	Myoblast differentiation	miRNA-125b and miR-351-5p	Du J., et al, 2019;
lnc-mg	Myoblast differentiation	*MYMK*	Militello, G., et al, 2018
lncMUMA	Myoblast differentiation	miR-762	Zhang Z.K. et al, 2018
lncMyoD	Myoblast differentiation	*IMP2*	Gong C. et al, 2015
lncMyoD	Myoblast differentiation	miR-370-3p	Zhang P. et al, 2021
MAR1	Myoblast differentiation	miR-487b	Zhu M., et al, 2017
Meg3	Muscle development	TGFβ signalling	Dill T.L. et al, 2021
MUNC	Myoblast differentiation	*MYOD*, *MYOG* and *MHC3*	Mueller A., et al, 2015
Myolinc	Myoblast differentiation	TDP43 and Filip1	Militello G. et al, 2018
Myoparr	Myoblast differentiation	Ddx17/P300/PCAF	Hitachi K., et al, 2019
Sirt1 AS	Myoblast proliferation	miR-34a	Wang G.Q. et al, 2016
SRA	Myoblast differentiation	*MYOD*	Caretti G., et al, 2006
SYISL	Myoblast proliferation	PCR2	Jin J.J. et al, 2018
yylncT	Mesodermal differentiation	T	Frank S. et al, 2019

Table 1. Summary of the lncRNAs and their functions in the skeletal muscle: myoblast proliferation (light green); myoblast differentiation (green); muscle regeneration (dark green); muscle atrophy is (red); muscle development (light blue).

1.3.4.1. MALAT1

Metastasis associated lung adenocarcinoma transcript 1 (MALAT11) gene, also known as nuclear-enriched abundant transcript 2 (NEAT2) contains more than 8 kb and is located within human chromosome 11q13. MALAT1 displays a high level of conservation throughout 33 mammalian species and is ubiquitously expressed in almost all human tissues [Ji P. et al, 2003]. MALAT1 transcript is around 7-kb in humans and lacks a poly A tail.

The primary transcript of MALAT1 is processed by RNase P and RNase Z into a long 6.7-kb transcript, which localizes to nuclear speckles, sub-nuclear bodies containing pre-mRNA processing proteins, and a much smaller MALAT1-associated small cytoplasmic RNA (mascRNA) [Bernard D. et al, 2010].

MALAT1 was one of the first identified cancer-associated lncRNAs, since it drives tumorigenesis through promoting tumour cell proliferation [Zhang X. et al, 2017].

However, several studies demonstrated a crucial and relevant role of this molecule in the myogenic processes. Indeed, MALAT1 was found up-regulated during differentiation of both human and murine myoblasts [Watts R. et al, 2013; Butchart L.C. et al, 2016]. Of note, MALAT1 modulates Serum response factor (SRF) through miR-133 via ceRNA mechanism, thus stimulating myocyte differentiation [Han X. et al, 2016]. Moreover, MALAT1 inhibition reduces C2C12 myoblast proliferation and promote their differentiation into myotubes, acting on the expression of *MYOG* gene [Watts R. et al, 2013]. A more recent

study showed that inhibition of MALAT1 accelerates the myogenic differentiation *in vitro*. These experiments were confirmed by *in vivo* studies, where MALAT1 KO mice displayed a significant muscle regeneration after injury and in dystrophic mice. According to the authors, *MYOD* is inhibited by MALAT1 but, upon differentiation, the pro-myogenic miR-181 expression level is increased and it targets MALAT1 transcripts for degradation, allowing *MYOD* activation [Chen X. et al, 2017]. Another *in vivo* study showed that MALAT1 expression levels are not altered by a variation of physical activity and muscle mass [Hitachi K. et al, 2020]. In addition, it was also observed that the expression of MALAT1 was significantly upregulated throughout the skeletal muscle regeneration process *in vivo*, positively correlating with the expression of *MYOG* and *MYOD*. These findings supported the hypothesis that Malat1 is implicated in the regeneration of contused skeletal muscle [Zheng L. et al, 2020].

1.3.4.2. NEAT1

Nuclear enriched abundant transcript 1 (NEAT1), also known as Men ε/β, is transcribed from the same promoter of MALAT1, generating a short variant called NEAT1_v1 or MENε of 3.7 kb transcript, and a longer one, called NEAT1_v2 or MENβ, of 23 kb. NEAT1_v1 RNA is polyadenylated, whereas NEAT1_v2 is not, since it is an essential component for the formation of the nuclear structure paraspeckles, harbouring a critical role in the control of gene expression of hyper-edited transcripts by tethering them within the nuclear bodies [Nishimoto Y. et al, 2021].

NEAT1_v2 interacts with core paraspeckle proteins PTB-associated splicing factor (PSF) and Non-POU domain containing octamer binding

(NONO), which recruit PSP1 and NEAT1_v1 and other associating molecules to the periphery of paraspeckles [Nakagawa S. et al, 2016].

As MALAT1, NEAT1 is highly conserved across mammalian species [Yamazaki T. et al, 2015].

In cattle muscle tissue, the expression levels of NEAT1 are upregulated during the development of the muscle tissue suggesting that this upregulation is associated by an increased number of nuclei in the developing myofibers [Lehnert S.A. et al, 2007]. However, a higher NEAT1 expression was observed in C2C12 cells upon myoblast differentiation, because of NEAT1 direct interaction with NONO [Sunwoo H. et al, 2009]. In line with this, in a C57BL/6J mouse model it was observed that both variants of NEAT1 are upregulated during post-natal growth, with NEAT1_v2 showing a more gradually increase than NEAT1_v1 [Butchart L.C. et al, 2016]. Moreover, analysing a pig model of acute physical activity, it was reported that NEAT1 was upregulated in this stressing condition, probably in the attempt to regenerate the muscle tissue [Jensen J.H. et al, 2012].

In a conflicting study, NEAT1 expression levels did not show any significant alteration in atrophic and hypertrophic mouse models, suggesting that NEAT1 involvement in skeletal muscle development is not completely clear [Hitachi K. et al, 2020].

However, the main suggested function for NEAT1 is in regulating myoblast proliferation, rather than differentiation, considering its role in the generation and development of the muscle tissue. Indeed, a more functional study showed that knockdown of NEAT1 improved the cross-sectional area of muscle fibres by increasing the expression of myogenic genes and delaying muscle regeneration, primarily by a reduction in the number of $Pax7^+$ cells. Specifically, NEAT1 promotes myoblast proliferation and inhibits myogenic differentiation by guiding EZH2 to target *MYOG*, *MHC4*, Troponin I2 (*Tnni2)*, and CDKN1A

cyclin-dependent kinase inhibitor 1A (*p21)* genes. Moreover, NEAT1 regulate myogenesis via other proteins or signalling pathways, because NEAT1 also inhibited *MYOD* expression in an EZH2-independent manner [Wang S. et al, 2019b].

1.3.4.3. HOTTIP

HOXA transcript at the distal tip (HOTTIP) is a lncRNA of 3.7 kb, transcribed from the 5' tip of the Homeobox A Cluster (*HOXA*) locus on chromosome 7, that coordinates the activation of several 5' HOXA genes *in vivo*.

In the skeletal muscle, the chromosomal looping brings HOTTIP into close proximity to its target genes, where it binds the adaptor protein WD Repeat Domain 5 (WDR5) and WDR5/ Mixed lineage leukemia protein-1 (MLL) complexes, driving histone H3 lysine 4 trimethylation (H3K4me3) and thus causing gene transcription [Wang K.C. et al, 2011]. In mouse and chick embryos, it was shown that HOTTIP is expressed in posterior and distal anatomical locations, suggesting a conserved HOTTIP expression pattern from development to adulthood [Wang K.C. et al, 2011]. Moreover, HOTTIP is required to maintain active expression of 5' HOXA genes, by keeping a stable MLL complex at the 5' end of HOXA gene cluster [Pradeepa M.M. et al, 2017]. HOTTIP expression is required for the activation of *HOXA13* and *HOXA10/11* and, at a lower level, of *HOXA9* and *HOXA7* [Wang K.C. et al, 2011]. KO HOTTIP *in vivo* experiments in the mouse showed abnormalities of the hind limbs, including muscle weakness and skeletal malformations, suggesting a central role for this molecule in the development of the skeletal muscle [Lai K.M. et al, 2015].

1.3.5. LncRNAs in muscle and motor neuron diseases

Growing evidence highlights the role of lncRNAs as regulators of muscle mass as shown in Table 1. In the field of muscle diseases, the role of lncRNAs in the atrophic processes was investigated in skeletal muscle disease as Duchenne muscle atrophy (DMD).

It was demonstrated that lnc-31 counteracts myoblast differentiation and its expression is abundant in human DMD myoblasts, thus actively participating in the disease progression by the interaction with Rho associated coiled-coil containing protein kinase 1 (ROCK1) [Ballarino M. et al, 2018; Dimartino D. et al, 2018]. In addition, it was shown that lncRNAs reduce the regenerative capacity of the skeletal muscle stimulating muscle hypertrophy and atrophic pathways. Indeed, it was demonstrated that LINC00961 is downregulated in mouse muscle upon acute injury, since it interacts with lysosomal v-ATPase negatively regulating mTOR Complex 1 (mTORC1) activation, acting as suppressor of muscle regeneration ability [Matsumoto A. et al, 2016]. Chronos, that is positively regulated in muscle with advancing age, has the ability to inhibit muscle hypertrophy by zeste 2 polycomb repressive complex 2 subunit (EZH2) interaction regulating muscle gene expression [Neppl R.L. et al, 2017]. Furthermore, Atrophy-related long noncoding RNA-1 (Atrolnc-1) is abundantly expressed in skeletal muscle and its expression is markedly increased in atrophying muscles from mice with cachexia. Mechanistically, Atrolnc-1 strongly binds to ABIN-1 inhibiting NF-κB signalling and causing protein degradation in muscle cells [Sun L. et al, 2018].

In the field of MN diseases, the role of lncRNAs is not completely clear and investigated. However, different lncRNAs were shown to interact with TDP43, with only two studies deepening their role in myogenesis [Li P. et al, 2015; Militello G. et al, 2018; Keihani S. et al, 2019; Li J. et al.

2020]. However, their involvement in ALS pathological processes still needs to be uncovered.

As demonstrated in C2C12 cells, Myolinc is an important regulator of myogenic differentiation *in vitro* and it is involved in skeletal muscle regeneration *in vivo*, since Myolinc siRNA injection in mouse tibialis anterior determines a reduction in fibre cross sectional area and in MHC^{+} myofibers. In fact, Myolinc−TDP43 interaction was shown to be essential for the binding of TDP43 to the promoter regions of *MYOD* and other muscle marker genes to control signalling pathways necessary for the differentiation of myoblasts into myofibers [Militello G. et al, 2018]. Another TDP43 binding lncRNA H19 acts a scaffold to localise TDP43 at the promoter region of *MYOD* in porcine SCs, thus promoting myogenic differentiation [Li J. et al. 2020].

In ALS patients it was observed that NEAT1_v2, the essential component of the paraspeckles, is highly enriched in neurons of the anterior horn of the spinal cord and in cortical tissues. In line with this, paraspeckle hyper-assembly was also observed in ALS [Shelkovnikova T.A. et al, 2018]. At the cellular level, NEAT1_v2 colocalizes with TDP43 and FUS proteins in ALS MN paraspeckles [Nishimoto Y. et al, 2013]. However, the role of NEAT1_v2 and paraspeckles in ALS progression remains poorly understood. It was only hypothesised that NEAT1_v2 facilitates paraspeckle formation, as a protective response in cells with impaired function of the mRNA machinery [Shelkovnikova T.A. et al, 2018].

Two antisense transcripts were found to be transcribed from *C9ORF72* gene: C9ORF72 sense (C9ORF72-S) and antisense (C9ORF72-AS). Both transcripts are translated into poly-dipeptides and found in the MNs of C9ORF72 ALS patients [Mori K. et al, 2013]. Moreover, the C9ORF72-S and C9ORF72-AS form RNA foci in the spinal cord of ALS patients. Poly-dipeptides and RNA foci represent

two distinct ways in which C9ORF72 repeat expansion evoke neurotoxic effects, suggesting that the two lncRNAs contribute to ALS pathogenesis [Gendron T.F. et al, 2013].

Ataxin 2 (*ATXN2*) is a coding gene linked to ALS [Elden A.C. et al, 2010]. ATXN2 localizes in the endoplasmic reticulum and is implicated in different functions, as translation, mRNA maturation, energy metabolism, endocytosis and RNA-binding protein [Sproviero W. et al, 2017]. An antisense transcript from the *ATXN2* locus, ATXN2-AS, was also described. As for C9ORF72-S and C9ORF72-AS, ATXN2-AS has a neurotoxic effect in cortical neurons, because of its disrupting effect on RNA metabolism, contributing to ALS pathogenesis [Li P.P. et al, 2016].

In Spinal muscular atrophy (SMA) it was identified a lncRNA, Survival motor neuron antisense strand 1 (SMN-AS1), which represses *SMN* expression by recruiting PCR2 contributing to the selective MN degeneration in SMA disease. Moreover, SMN-AS1 targeted degradation with antisense oligonucleotides (ASOs) in neurons, improves survival of severe SMA mice [d'Ydewalle C. et al, 2017].

2 Materials and Methods

2.1. Patients

The study included: four ALS patients, one female and one male carrying the G_4C_2-rich *C9ORF72* repeat (OMIM#105550); two males, carrying the p.A382T mutation in *TARDBP* gene (OMIM#605078.0013); two age- and sex-matched healthy controls. The clinical features of all subjects included in the study are shown in Table 2.

Patient	Sex	Age at onset	Region of onset	Phenotype	sALS/fALS	Mutation
C9ORF72_1	F	46,9	S-LL	UMNP	sALS	*C9ORF72* repeat expansion
C9ORF72_2	M	61,2	S-LL	Classical	sALS	*C9ORF72* repeat expansion
TDP43_1	M	50,7	S-UL	Classical	sALS	p.A382T in *TARDBP*
TDP43_2	M	n/a	n/a	n/a	sALS	p.A382T in *TARDBP*
CTRL_1	F	45	n/a	n/a	n/a	n/a
CTRL_2	M	37	n/a	n/a	n/a	n/a

Table 2. Summary of the main features of ALS patients. F, female; M, male; S-LL, spinal-lower limb onset; S-UL, spinal-upper limb onset; UMNP, upper motor neuron predominant.

Written informed consent was obtained from ALS patients and from healthy controls. The study was approved by the local Ethics Committee and performed according to the amended Declaration of Helsinki.

2.2. Fibroblast cultures, iPSC generation and spontaneous differentiation into three germ layer-specific cell types

Fibroblasts were isolated from skin biopsies as previously described [Cazzato D. et al, 2016]. Cells were maintained and expanded until passage 3-4 in Dulbecco's modified Eagle medium (DMEM) supplemented with 20% Fetal bovine serum (FBS), 1% L-Glutamine, 1% non-essential amino acids, 100 mM sodium pyruvate and 1% penicillin/streptomycin (P/S). Fibroblasts were plated at a 30%-60% confluence and after two days they were infected by using CytoTune®-iPS 2.0 Sendai Reprogramming Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA) as previously described [Bardelli D. et al, 2020]. After the infection, they were maintained in their medium for 7 days and therefore plated onto Matrigel coated dishes (Corning, Glendale, AZ, USA). From day 8, medium was replaced with the iPSC-specific Essential 8 medium (Thermo Fisher Scientific). At day 14-15, six clones per sample were picked for expansion. Colonies were passaged using an EDTA 0.5 mmol/L solution (Euroclone, MI, Italy) and cultured in Essential 8 medium.

iPSC pluripotency was evaluated via assessing the ability of iPSCs to spontaneously differentiate into cells of the three germ layers, ectoderm, endoderm and mesoderm. iPSCs were suspended in low adhesion plates in HuES medium composed by DMEM/F12, 20% knock-out serum replacement, 2 mmol/L L-glutamine, 10 U/mL penicillin, 10 µg/mL streptomycin, 0.1 mmol/L Minimum Essential Medium (MEM) Non-Essential Amino Acids (NEAA) and 110 µmol/L β-mercaptoethanol (all reagents from Thermo Fisher Scientific). After 7

days, embryoid bodies (EBs) were generated. They were plated on Matrigel-coated coverslips in Essential 8 medium (Thermo Fisher Scientific) for additional 10 days to obtain cells differentiated into the three germ layers.

2.3. PCR analysis for pluripotency markers

iPSC characterization was performed to evaluate the pluripotency of the cells by PCR using primers specific for pluripotency-associated stem cell markers including SRY‐box transcription factor 2 (SOX2), Octamer binding transcription factor 3/4 (OCT 3/4) and Nanog homobox (NANOG) (Eurofins Genomics, Luxembourg, LU). Total RNA was isolated from iPSCs using Trizol Reagent (Thermo Fisher Scientific) following manufacturer's protocol, and reverse transcribed into cDNA (40 ng/uL) using SuperScript II reverse transcriptase (Thermo Fisher Scientific). PCR was performed with the following protocol: denaturation 95°C 30 seconds, annealing 60°C 30 seconds, elongation 72°C 1 minute, for 35 cycles and the primers are listed in Table 3.

Gene	Primer	Sequence
SOX2	F	GGGAAATGGGAGGGGTGCAAAAGAGG
SOX2	R	TTGCGTGAGTGTGGATGGGATTGGTG
OCT3/4	F	GACAGGGGGAGGGGAGGAGCTAGG
OCT3/4	R	CTTCCCTCCAACCAGTTGCCCCAAC
NANOG	F	CAGCCCTGATTCTTCCACCAGTCC
NANOG	R	GTTCTGGAACCAGGTCTTCACCTG

Table 3. Sequences of the primers used for PCR.

2.4. Generation of human skeletal muscle cells

Skeletal muscle cells were obtained by skeletal muscle differentiation protocol adapted from Swartz and colleagues [Swartz E. W. et al, 2016]. The iPSCs were cultured for 1.5 days in Essential 8 medium (Thermo Fisher Scientific) supplemented with 1% dimethyl sulfoxide (DMSO) (Merck, RM Italy) to select the cells [Ogaki S. et al, 2015]. Then, the medium was replaced with Chemically defined medium supplemented with bFGF, Ly294002, BMP4 and CHIR99021 (CDMFLyBC). CDM was composed of DMEM/F12 medium supplemented with bovine serum albumin (BSA) (Merck), chemically defined (CD) lipid concentrate 0.01% (Thermo Fisher Scientific), transferrin 15 ug/mL (Merck), 1-thioglycerol 450 uM (Merck), insulin 7 ug/mL (Thermo Fisher Scientific), 0,01% P/S (Thermo Fisher Scientific). CDM medium was supplemented with 20 ng/mL of basic fibroblast growth factor (bFGF) (Peprotech, Rocky Hill, NJ, USA), 10 uM of 2-(4-Morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (Ly294002) (MedChem, Monmouth Junction, NJ, USA), 10 ng/mL of bone morphogenetic protein 4 (BMP4) (Peprotech) and 10 uM of 6-[[2-[[4-(2,4-Dichlorophenyl)-5-(5-methyl-1H-imidazol-2-yl)-2-pyrimidinyl]amino]ethyl]amino]-3-pyridinecarbonitrile (CHIR99021) (StemCell Technologies, Vancouver, Canada). Cells were cultured in CDMFLyBC for 1.5 days. At day 1.5 medium was changed to CDMFLy, consisting of CDM supplemented with 20 ng/mL of bFGF and 10 uM of Ly294002 until day 7. At day 7, the cells were cultured in Myoblast Growth Medium (MGM) composed by 75% of Ham's F-12 Nutrient Mix (Thermo Fisher Scientific) and 25% of FBS (Thermo Fisher Scientific), supplemented with 2.5 ng/mL of bFGF (resuspended in 0.5% BSA/PBS solution) and 1% P/S. After 5 days, the medium was changed to fusion medium (FM), made of DMEM and 2% horse serum (Merck) (Thermo Fisher Scientific), and it was fully changed on alternating days.

To obtain mature myotubes, the medium was replaced with the terminal differentiation medium (TDM), composed of DMEM/F12 (Thermo Fisher Scientific) plus N2-supplement (1%) (Thermo Fisher Scientific) and ITS Liquid Media Supplement (1%) (Merck) and fully changed daily for 14 days.

2.5 ALS mutation analysis

Genomic DNA was extracted from IPSCs and iPSC-derived myotubes, according to a standard phenol-chloroform procedure. DNA samples were screened for exon 6 of *TARDBP* gene. Exon and intron–exon boundaries were amplified by polymerase chain reaction (PCR) and analysed by direct sequence analysis using an automated sequencing system (ABI 3130 XL, Thermo Fisher Scientific). Nucleotides and amino acid residues were numbered according to the reference gene sequence of the transcript GenBank (NCBI).

The GGGGCC hexanucleotide repeat in *C9ORF72* gene was analysed by a two-step protocol, including a first PCR amplification step using the genotyping primers previously reported [DeJesus-Hernandez M. et al., 2011]. The normal range fragment length analysis was performed on 2% agarose gel. Samples presenting two distinct amplification products in the normal size range are definitively considered negative. Samples resulting in a single amplification product were further analysed in a second step by the repeat-primed polymerase chain reaction method (AmplideX PCR/CE C9orf72 Kit (RUO)) on a 3100XL ABI Prism Genetic Analyzer (Applied Biosystems). The presence of C9ORF72 repeat expansion (RE) was assigned when the sample displayed a typical electropherogram profile with decaying stutter amplification peaks. This method are highly sensitive and robust, single-tube, 3-primer C9ORF72 PCR reagents that can flag all

expanded samples irrespective of length and provide accurate sizing up to ~145 repeat units using capillary electrophoresis.

2.6. Real-time PCR for myogenic marker expression

Total RNA was extracted by TRIzol reagent and reverse-transcribed using SuperScript VILO cDNA Synthesis kit (Thermo Fisher Scientific Inc.). cDNA (10 ng) was amplified in duplicate by Taqman gene expression assays specific PAX3, PAX7, MYOD1, MYOG, desmin (DES), MHC2, MHC3, MHC7 on the ViiA7 Real time PCR system (Thermo Fisher Scientific). 18s ribosomal RNA was stably expressed in both control and ALS samples (standard deviation of Ct values: < 0.5) and was used as endogenous control. Transcriptional levels of the target genes were expressed as relative values normalized towards 18s levels according to the following formula $2^{-\Delta Ct}$.

2.7. Immunofluorescence

Immunostaining analysis was performed on cells obtained from the EBs, iPSCs, and iPSC-derived myotubes plated onto Matrigel-treated glass coverslips. The cells were fixed in 4% paraformaldehyde at room temperature for 20 minutes, permeabilized with 0.25% Triton X-100 (Carlo Erba Reagents, Cornaredo, MI, Italy) for iPSCs and 0.1 %Triton X-100 for cell from the EBs and cells differentiated into myotubes, and treated with 10% normal goat serum (NGS, Vector Laboratories, Burlingame, CA, USA) in PBS.

Cells derived from the EBs were incubated with the following antibodies: III β-tubulin (β-III-TUB) (1:500, rabbit monoclonal, Abcam) for ectoderm, DES (1:10, rabbit polyclonal, Merck, Darmstadt, Germany) for mesoderm and Alpha-fetoprotein (AFP) (1:125, mouse monoclonal, Thermo Fisher Scientific) for endoderm.

To analyse the pluripotency of iPSCs, these cells were stained for 90 minutes at 37°C with the following primary antibodies: T cell receptor alpha locus (TRA) -1-60 (1:125, mouse monoclonal, Thermo Fisher Scientific), Stage-specific embryonic antigen-4 (SSEA-4) (1:100, mouse monoclonal, Thermo Fisher Scientific) and alkaline phosphatase (ALP) (1:250, rabbit monoclonal, Abcam, Cambridge, UK).

The iPSC-derived myotubes were immunostained over night at 4°C with: rabbit anti-T (1:1000, rabbit polyclonal, Abcam), rabbit anti-PAX3 (1:200, rabbit polyclonal, Abcam), mouse anti-PAX7 (1:200, mouse monoclonal, Abcam), rabbit anti-MYOD1 (1:200, rabbit polyclonal, Abcam), mouse anti-MYOG (1:200, mouse monoclonal, Thermo Fisher Scientific), rabbit anti-MHC2 (1:2, rabbit monoclonal, Leica Biosystems, Wetzlar, Germany) and rabbit-anti DES (1:200, rabbit polyclonal, Bio-Techne, Minneapolis, MN, USA). These cells were also stained with hematoxylin-eosin (Bio Optica, Milan, Italy) and examined by optical microscopy (Eclipse E100, Nikon).

Immunopositivity was revealed with Alexa Fluor 488-conjugated goat anti-mouse IgG, Alexa Fluor 555-conjiugated goat anti-rabbit IgG, and Alexa Fluor 546-conjugated goat anti-rabbit IgG secondary antibodies (All antibodies 1:500, Thermo Fisher Scientific). Cells were counterstained with DAPI (1:1000) (Thermo Fisher Scientific) and the coverslips were mounted with FluorSave (Merck). Confocal fluorescence images were obtained with a laser-scanning microscope (Eclipse TE 2000-E, Nikon, Tokyo) and analysed using EZ-C1 3.70 imaging software (Nikon).

2.8. Real-time PCR for lncRNA expression

We selected three lncRNAs, MALAT1, NEAT1 and HOTTIP from literature, chosen because they were specifically expressed in the muscle tissue and associated with development and maintenance of

the skeletal muscle. cDNA previously analysed for myogenic marker expression, was amplified in duplicate by TaqMan gene expression assays (Thermo Fischer Scientific) specific for MALAT1, NEAT1 and HOTTIP on the ViiA7 Real time PCR system (Thermo Fisher Scientific). 18s ribosomal RNA was stably expressed in both control and ALS samples (standard deviation of Ct values: < 0.5) and was used as endogenous control. Transcriptional levels of lncRNAs were expressed as relative values normalized towards 18s levels, according to the following formula $2^{-\Delta Ct}$.

2.9. Prediction and selection of lncRNA target genes

We aimed to predict *in silico* the target genes of MALAT1, NEAT1 and HOTTIP, in order to identify potentially disease-modifying pathways and to investigate their regulatory networks in the skeletal muscle tissue. We used two online resources, lncTarD [http://biocc.hrbmu.edu.cn/LncTarD/JumpToAdvancedSearch#tableDivHead] and LncRNA2Target v2.0 [http://123.59.132.21/lncrna2target/search.jsp], to predict functional lncRNA target genes. Network analysis was performed via the use of Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases [Gene Ontology Consortium 2013; Kanehisa M. et al, 2000]. Enrichment analyses of the identified potential target genes were performed using ClueGO app implemented in Cytoscape v3.7.1 to identify the possible implications of the dysregulated lncRNAs and mRNAs in the relevant GO terms and signalling pathways [Shannon P. et al, 2003]. Bonferroni adjusted P-value <0.05 was considered to indicate a statistically significant difference. Heat map analysis of lncRNA and mRNA expression profiles was performed using the Pheatmap R package.

2.10. Real-time PCR for lncRNA target genes

cDNA previously analysed for myogenic markers and lncRNA expression, was amplified in duplicate by TaqMan gene expression assays (Thermo Fisher scientific) specific for AKT, Autophagy related (ATG) 4D, ATG7, ATG12, B cell lymphoma-2 (BCL2), Caspase (CASP) 3, CASP9, DEAD-box helicase 5 (DDX5), ELAV like RNA binding protein 1 (ELAVL1/HUR), EZH2, High mobility group box 1 (HMGB1), HOXA11, HOXA13, Mitogen-activated protein kinase (MAPK) 1, MAPK3, Mouse double minute 2 (MDM2), mTOR, MYOG, Nuclear factor kappa-light-chain-enhancer of activated B cells (NFKB1), Ras-related protein RAB5A (RAB5A), Suppressor of cytokine signalling 1 (SOCS1), Signal transducer and activator of transcription 3 (STAT3), TGFβ1, Transforming growth factor beta receptor (TGFβR) 1, TGFβR2 and WD repeat domain 5 (WDR5) on the ViiA7 Real time PCR system (Thermo Fisher Scientific). 18s ribosomal RNA was stably expressed in both control and ALS samples (standard deviation of Ct values: < 0.5) and was used as endogenous control. Transcriptional levels of lncRNA target genes were expressed as relative values normalized towards 18s levels according to the following formula $2^{-\Delta Ct}$.

Gene	**Assay ID** (Thermo Fisher)
ATG4D	Hs00262792_m1
ATG7	Hs00893766_m1
ATG12	Hs04980076_s1
BCL2	Hs04986394_s1
CASP3	Hs00234387_m1
CASP9	Hs00962278_m1
DDX5	Hs01075383_g1
DES	Hs00157258_m1

ELAVL1/HUR	Hs00171309_m1
HMGB1	Hs01923466_g1
HOXA11	Hs00194149_m1
HOXA13	Hs00426284_m1
MAPK1	Hs01046830_m1
MAPK3	Hs00385075_m1
MDM2	Hs00540450_s1
MHC2	Hs00430042_m1
MHC3	Hs01074230_m1
MHC7	Hs01110632_m1
mTOR	Hs00234508_m1
MYOD	Hs00159528_m1
MYOG	Hs01072232_m1
NFKB1	Hs00765730_m1
PAX3	Hs00240950_m1
PAX7	Hs00242962_m1
RAB5A	Hs00702360_s1
SOCS1	Hs00705164_s1
STAT3	Hs00374280_m1
TGFβ1	Hs00998133_m1
TGFβR1	Hs00610320_m1
TGFβR2	Hs00234253_m1
WDR5	Hs00424605_m1

Table 4. IDs of the assays used for RT-PCR.

Results 3

3.1. Generation of iPSCs from CTRL, C9ORF72 and TDP43 patients

We generated iPSC lines by reprogramming fibroblasts from two C9ORF72 and two TDP43 mutant ALS patients and two age- and sex-matched healthy controls. Both ALS and control iPSCs expressed the pluripotency markers including SOX2, OCT3/4 and NANOG (Figure 5.A.). In addition, the cells were positive to ALP, SSEA-4 and TRA-1-60 (Figure 5.B.). These molecules are specific markers for undifferentiated pluripotent stem cells, thus indicating that the iPSCs showed a pluripotent state [Takahashi K. et al, 2006]. All ALS and control iPSCs displayed similar morphology and proliferative capacity (Data not shown). Next, we investigated the iPSC pluripotency capacity by assessing their ability to spontaneously differentiate into cells of the three germ layers [Takahashi K. et al, 2006]. All iPSCs formed EBs which directly differentiated into ectoderm, endoderm and mesoderm positive to β-III-TUB, AFP and DES, respectively (Figure 5.C.). All reprogrammed iPSCs expressed pluripotency markers, differentiated into the three germ layers and did not show any significant difference in morphology.

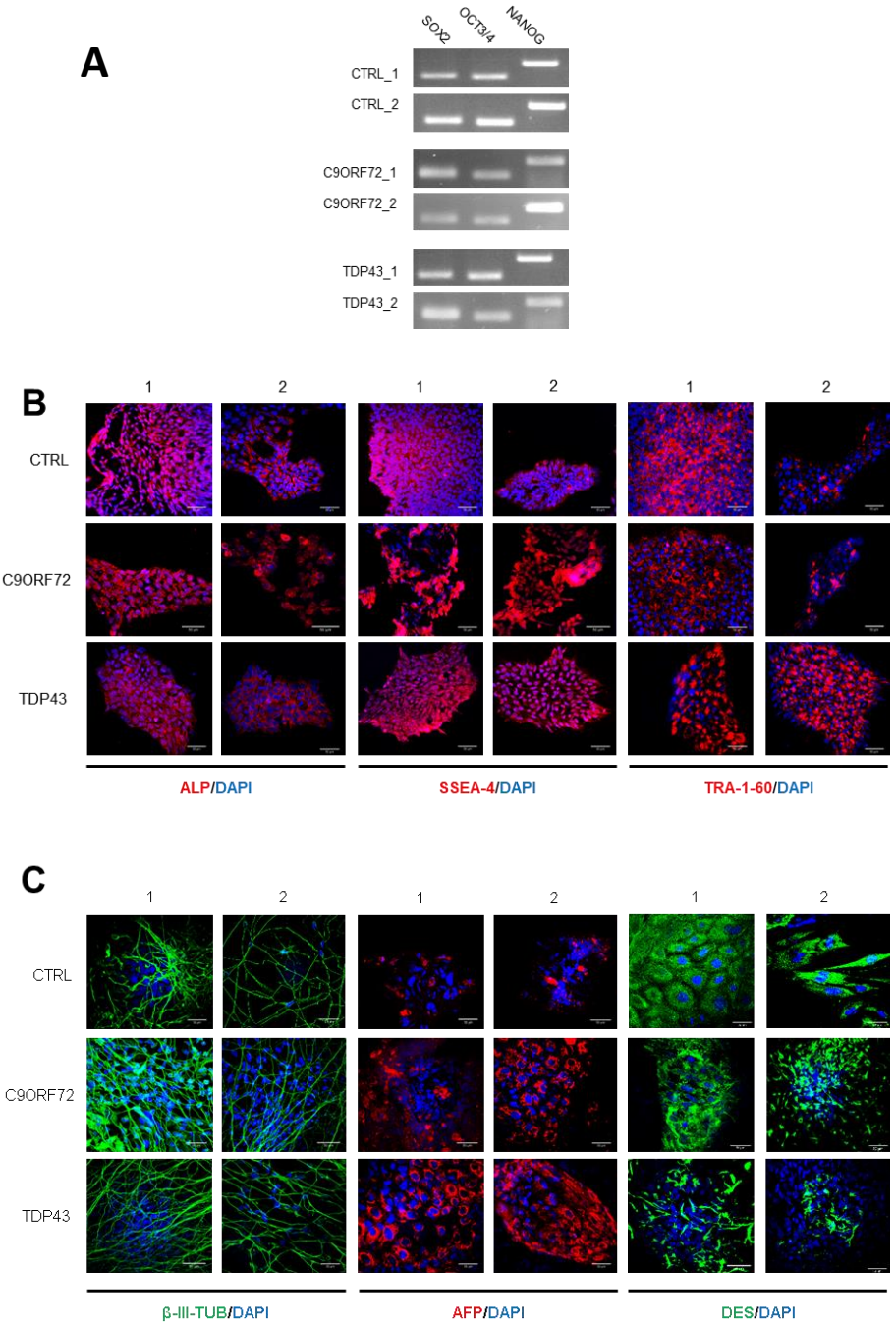

Figure 5. Generation of iPSC colonies. A. RT-PCR analysis of SOX2, OCT3/4 and NANOG genes in CTRL, C9ORF72 and TDP43 iPSCs (N=2 per group). B. Representative confocal images of CTRL, C9ORF72 and TDP43 iPSCs positive for ALP (red), SSEA-4 (red), TRA-1-60 (red). Cell nuclei were stained with DAPI (blue). Scale bar, 50 μm. C. Representative confocal images of CTRL, C9ORF72 and TDP43 iPSCs stained for β-III-TUB (green), AFP (red) and DES (green) and DAPI (blue). Scale bar, 50 μm.

3.2. Generation of contractile myotubes from CTRL, C9ORF72 and TDP43 iPSCs

To obtain skeletal muscle cells, we differentiated ALS and control iPSCs into spontaneously beating myotubes, after 36 days of differentiation. Figure 6. shows different myogenic differentiation stage of control cells from the CTRL_1 line stained with haematoxylin/eosin.

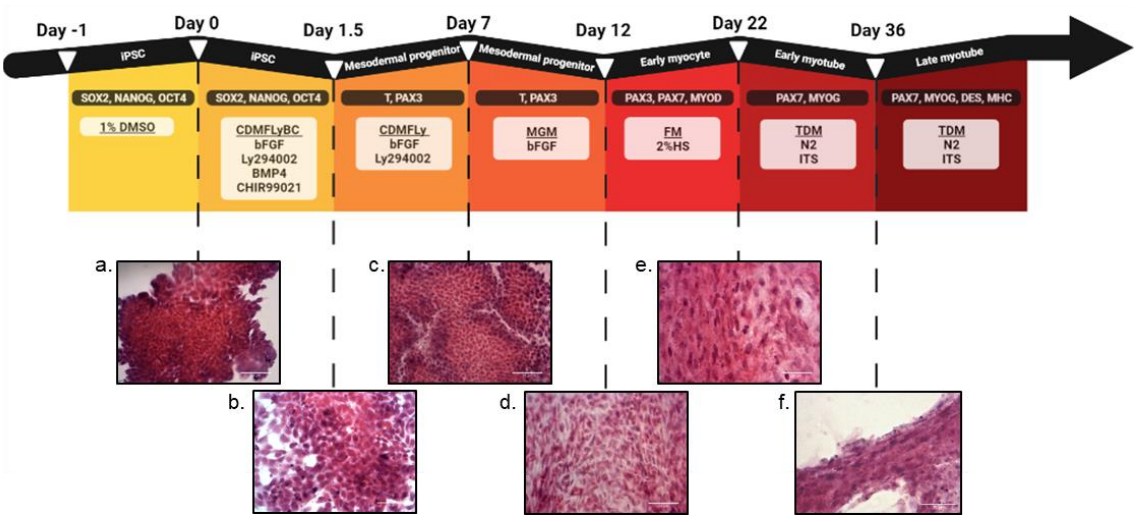

Figure 6. Timeline diagram of myogenic differentiation protocol for obtaining spontaneously contractile myotubes. Marker genes and medium composition are represented for each stage in black or white squares, respectively. The lower panel shows representative hematoxylin/eosin stained CTRL_1 cells at day 0 (a.), day 1.5 (b.), day 7 (c.), day 12 (d.), day 22 (e.) and day 36 (f.)40x magnification.

At day 0 the cells showed the typical iPSC morphology positive to SOX2, NANOG and OCT4, with very large nuclei, little cytoplasm and colonial organization (Figure 6.a.) [Nagasaka R. et al, 2017]. After 1.5 days in CDMFLyBC medium, cells started to migrate towards the edges of the colony and were increasing in size. At the same time, cells located at the periphery of the colony acquired a more defined morphology, with distinct edges; indeed, the central ones were still

undifferentiated, retaining a roundish shape (Figure 6.b.). From day 1.5 to day 7, cells completely abandoned the colonial organization, generating a unique layer, and are positive to T and PAX3 myogenic markers. Moreover, cells left their undifferentiated architecture and their morphology shifted from roundish to polygonal, but still retaining minimal amounts of cytoplasm (Figure 6.c.). From day 7, the cells, besides differentiating, they actively proliferated, reaching the confluence in MGM at day 12. From that time, the cells are positive to PAX3, PAX7 and MYOD, showing a cellular morphology characterised by large and round nuclei, randomly and sparse distributed, comparable to an immature myoblast stage, became more phenotypically differentiated [Ishikawa K. et al, 2019]. In addition, they started to move in close proximity one to each other, to align and elongate (Figure 6.d.). At day 22, the cells positive to PAX7 and MYOG were morphologically similar to myocytes with a very low nuclear-cytoplasmic ratio, distinctive elongated nuclei surrounded by huge amounts of cytoplasm, and they started to fuse to become myotubes (Figure 6.e.) [Ishikawa K. et al, 2019].

At day 36, we observed multinucleated myotubes positive to PAX7, MYOG, DES and MHC myogenic markers, characterised by peripheral elongated nuclei, mixed with central roundish nuclei, still in differentiation [Ishikawa K. et al, 2019]. Moreover, the culture showed an organized architecture, where the myotubes arranged in a parallel, dense, fibre-like structure (Figure 6.f.). As shown in Video 1, at day 36 the myotubes showed a spontaneous contractive activity.

https://drive.google.com/file/d/1pGqquatbgqux3EouS0eTvN_n9J9Jophr/view?usp=sharing

Video 1. Spontanously beating myotubes- CTRL_1 beating myotubes after 36 days of myogenic differentiation. 10x magnification.

Of note, the iPSCs and iPSC-derived myotubes retained the G_4C_2-rich *C9ORF72* repeat (Figure 7.A.) and the *TARDBP* mutation (Figure 7.B.) of ALS patients, confirming that the cells after reprograming and muscle differentiation still expressed the specific genetic profile of the patients.

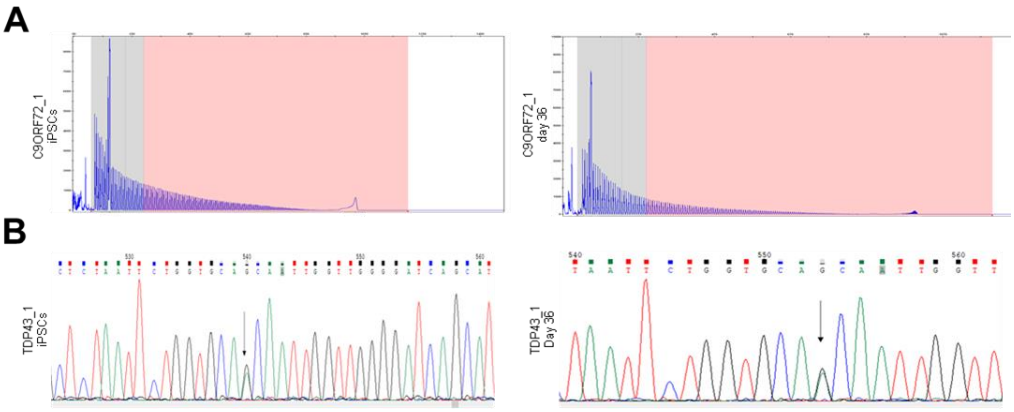

Figure 7. ALS related mutation retention in C9ORF72 and TDP43 cells. A. Repeat primed PCR from C9ORF72_1 to confirm the G_4C_2-repeat expansion in iPSCs (left panel) and at day 36 of differentiation (right panel). B. Sanger sequencing of p.A382T mutation in TDP43_1 in iPSCs (left panel) and at day 36 of myogenic differentiation (right panel).

3.3. Altered expression of myogenic markers in iPSC-derived myotubes from CTRL, C9ORF72 and TDP43 patients

To investigate the potential alterations in myogenic processes caused by ALS mutations, we analysed the expression levels of myogenic differentiation markers at different stages of skeletal muscle development, specifically at iPSC (day 0), mesodermal progenitor (day 12), early myocyte (day 22) and myotube (day 36) stages of differentiation. We evaluated the expression levels of two mesodermal

progenitor markers, T and PAX3. PAX3 is also an early myocyte marker, together with MYOD1.

Moreover, we investigated the expression levels of *MYOG* and *PAX7*, early myotube and satellite cell (SC) markers. The expression of *DES*, *MHC2*, *MHC3* and *MHC7*, late myotube markers was explored too (Figure 8.) [Zammit P.S. et al, 2006].

At day 0, the iPSCs did not express high levels of the myogenic markers in all cell cultures (Figure 8).

By molecular analyses, we observed an increased expression trend of *T, PAX3, PAX7, MYOD* and *MYOG* at day 12 compared to days o, 22 and 36. However, the expression of these markers showed a decreased expression trend in ALS cells compared to healthy controls. Among the disease lines, we detected a higher expression level of these markers in TDP43 cell lines than from the C9OFR72 ones (Figure 8.).

At day 22, we observed that *PAX3* expression was higher in CTRL than ALS cells, whereas it was lower in C9ORF72 lines than TDP43 ones. Moreover, *MYOD1* was expressed only in CTRL lines (Figure 8.).

At day 36, the expression levels of *PAX7, MYOD1, MYOG* showed a lower expression trend in ALS cells compared to controls, but among the two ALS lines, TDP43 cells showed a higher expression trend compared to C9ORF72 cells. Molecular data demonstrated the presence of active SCs, normally positive to *PAX7*, in all cultures. These data demonstrated that the iPSCs differentiated into SCs, representing a crucial stage for the myogenic development (Figure 8.) [Pradat P.F. et al, 2011]. In addition, at day 36, the molecular analyses revealed an increased expression of *DES, MHC2, MHC3* and *MHC7* compared to the other time points (day 0, day 12 and day 22),

suggesting that the differentiated cells showed a molecular signature of multinucleated myotubes (Figure 8.).

Indeed, comparing the expression of *DES* and *MHC3* at day 36, we detected a slightly lower expression of *DES*, in all cell lines, compared to the expression of *MHC3*, the embryonic myosin isoform. Specifically, CTRL and TDP43 lines showed a higher expression pattern of *DES* and *MHC3* than C9ORF72 lines (Figure 8.). At day 36 *MHC2* and *MHC7* the two mature myosin isoforms, showed an opposite expression trend. Indeed, *MHC2*, the fast myosin isoform, showed a lower expression trend in ALS lines compared to controls; whereas *MHC7*, the slow isoform, showed a higher expression trend in the ALS lines compared to controls, specifically in TDP43 compared to C9ORF72 cells (Figure 8.).

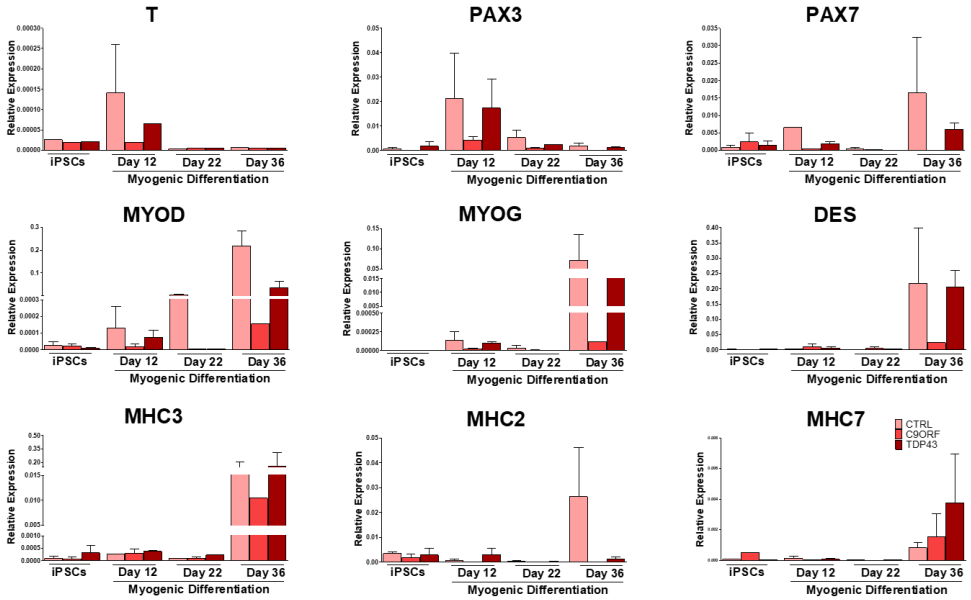

Figure 8. Transcriptional levels of the myogenic differentiation markers in iPSCs and iPSC-derived muscle cells at 12, 22, 36 days of differentation. qPCR analysis of *T, PAX3, PAX7, MYOD, MYOG, DES, MHC3, MHC2* and *MHC7* genes in CTRL (pink bar), C9ORF72 (red bar) and TDP43 (burgundy bar) cells at days 0, 12, 22 and 36 of myogenic differentiation protocol. Data were expressed as mean of two

patients for each group. Expression levels were normalized to 18S and expressed as fold changes using the 2−ΔCt formula.

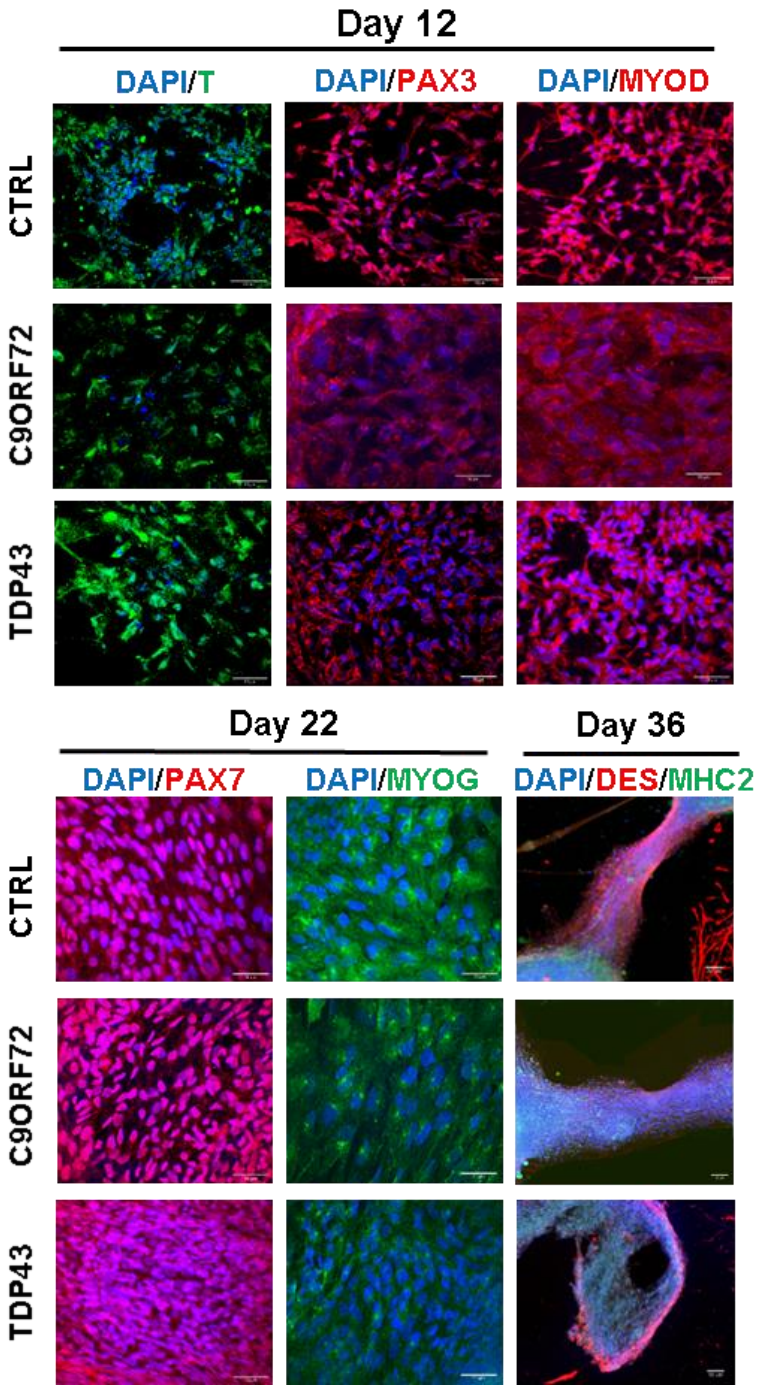

Figure 9. Differentation of iPSCs into myotubes. Representative confocal images of CTRL_2, C9ORF72_2 and TDP43_2 cells stained for myogenic differentiation

markers T (green), PAX3 (red), MYOD1 (red) and DAPI (blue) at day 12, for PAX7 (red), MYOG (green) and DAPI (blue) at day 22 and for DES (red), MHC (green) and DAPI (blue) at day 36. Scale bar, 50 µm.

By immunofluorescence, we found that iPSC-derived skeletal muscle cells were positive to T, PAX3 and MYOD1 at day 12, PAX7 and MYOG at day 22, and DES and MHC2 at day 36, as shown in Figure 9. Interestingly, at days 12 and 36 immunostaining data showed a different morphology for C9ORF72 cells compared to TDP43 and CTRL cell lines. In particular, at day 12 C9ORF72 cells displayed a higher amount of cytoplasm, as well as bigger nuclei, as shown by PAX3 and MYOD immunofluorescence markers (Figure 9.). At day 22, PAX7 staining revealed a delay in the elongation and parallelization of C9ORF72 cells, compared to TDP43 and CTRL cells. In addition, MYOG staining showed that C9ORF72 cells were characterised by a wider nucleus at day 22 (Figure 9.). However, at day 36 of differentiation ALS and CTRL differences were not detectable anymore from the morphological point of view. In fact, we reported an overlapping signal form DES and MHC2 positive cells from all cell lines (Figure 9.).

3.4. Dysregulated expression of lncRNAs in relation to human disease mutations and upon myogenic differentiation

To investigate whether lncRNAs have a role in muscle degeneration in ALS, we analysed the expression levels of selected lncRNAs, including MALAT1, NEAT1 and HOTTIP, that are known to be expressed in the skeletal muscle and play a relevant function in myogenic processes [Butchart L.C. et al, 2016; Chen X. et al, 2017; Han X. et al, 2015; Hitachi K. et al, 2020; Jensen J.H. et al, 2012; Lai K.M. et al, 2015; Lehnert S.A. et al, 2007; Pradeepa M.M. et al, 2017; Sunwoo H. et al, 2009; Wang K.C. et al, 2011; Wang S. et al, 2019b; Watts R. et al,

2013; Zhang X. et al, 2017; Zheng L. et al, 2019]. We investigated the lncRNAs in ALS and CTRL cell lines upon muscle differentiation (Figure 10.).

In ALS cells, MALAT1 expression trend is decreased at day 22 compared to controls (Figure 10.). In control cells the expression levels of MALAT1 showed an opposite trend; upon the early steps of differentiation at days 0, 12, and 22, MALAT1 expression progressively increased and it decreased at day 36 (Figure 10.).

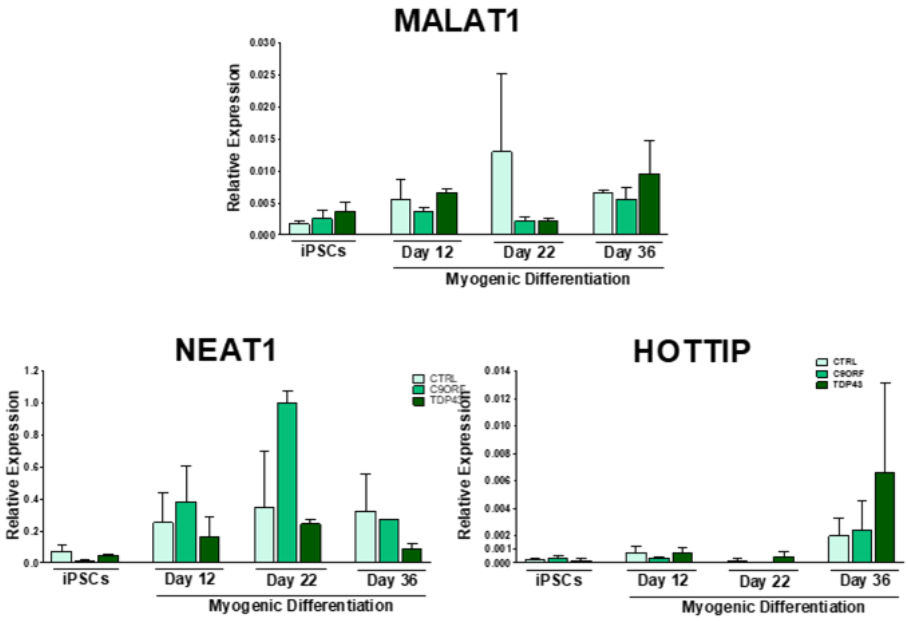

Figure 10. Expression of MALAT1, NEAT1 and HOTTIP lncRNAs upon the myogenic differentiation. qPCR analysis of lncRNAs MALAT1, NEAT1 and HOTTIP in CTRL (light yellow bar), C9ORF72 (yellow bar) and TDP43 (orange bar) cells at day 0, 12, 22 and 36 of myogenic differentiation protocol. Data were expressed as means of two patients for group. Expression levels were normalized to 18S and expressed as fold changes using the 2−ΔCt formula.

Regarding NEAT1, its expression trend gradually increased during the early stages of differentiation, particularly from the iPSC stage until day 22, in all cell lines, but it decreased at day 36.

TDP43 cell lines showed a lower expression trend of NEAT1, compared to CTRL and C9ORF72 lines, whereas mutant C9ORF72 cell lines exhibited a much higher expression trend compared to controls at day 12 and 22 (Figure 10.).

The expression trend of HOTTIP was increased during the myofibre maturation phase at day 36 compared to day 0, 12 and 22. Interestingly, both C9ORF72 and, even more, TDP43 mutant cell lines showed a higher expression trend of HOTTIP, compared to controls (Figure 10.).

These results suggest that the expression of the selected lncRNAs may contribute to skeletal muscle differentiation in relation to ALS pathogenesis.

3.5. MALAT1, NEAT1, HOTTIP and target gene involvement in signalling pathways associated to human disease mutations and myogenic differentiation

In order to understand the biological and molecular role of MALAT1, NEAT1 and HOTTIP in the skeletal muscle, by LncRNA2Target v2.0 and LncTArD databases and literature data, we identified and investigate 26 lncRNA target genes implicated in myogenic processes in relation to ALS pathogenesis (Figure 11.). In particular, we selected MALAT1's targets as key molecules for the regulation of the apoptosis (*BCL2, CASP3* and *CASP9*), autophagy (*ATG4D, ATG7, ATG12* and *RAB5A*), protein degradation/synthesis (*AKT1, MDM2* and *mTOR*), RNA editing (*ELAVL*), some miRNAs (miR-133 and miR-206) or

transcription factors (*EZH2, MYOG, HMBG1, MAPK1, MAPK3, NFKB1, SOCS1* and *STAT3*);

NEAT1's targets as relevant molecules for the modulation of apoptosis (*BCL2*), development (*TGFβ1*), RNA editing (*DDX5*), miRNAs (miR-124) and transcription factors (*EZH2, MYOG* and *STAT3*); HOTTIP's targets involved in apoptosis (*BCL2*), in development (*HOXA11, HOXA13, TGFβR1* and *TGFβR2*) and some transcription factors (*EZH2* and *WDR5*) (Figure 11.).

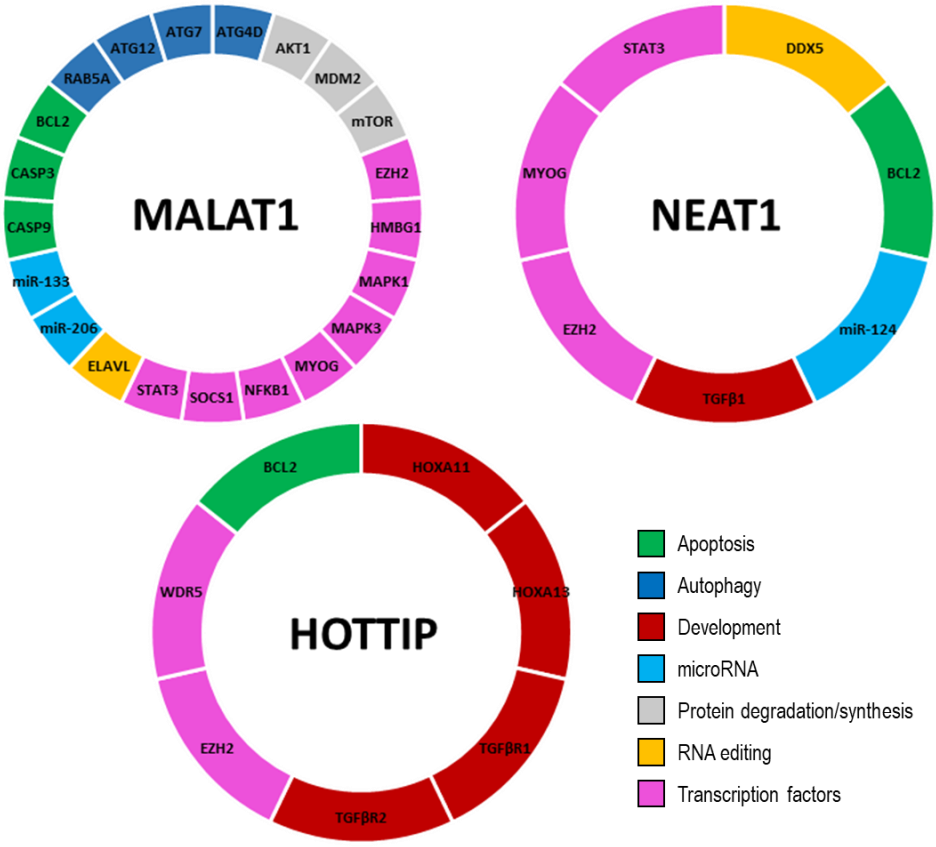

Figure 11. Cake graph representing the main functions of the predicted mRNA targets.

By hierarchical clustering analysis, we identified three distinct heat maps for C9ORF72, TDP43 and CTRL cell lines showing different

clusters that characterize iPSC and iPSC-derived muscle cells at day 12, day 22 and 36 stages of the differentiation (Figure 12.). Moreover, we distinguished a subcluster at day 12, which differentiated from another subcluster at day 22 and day 36 (Figure 12.).

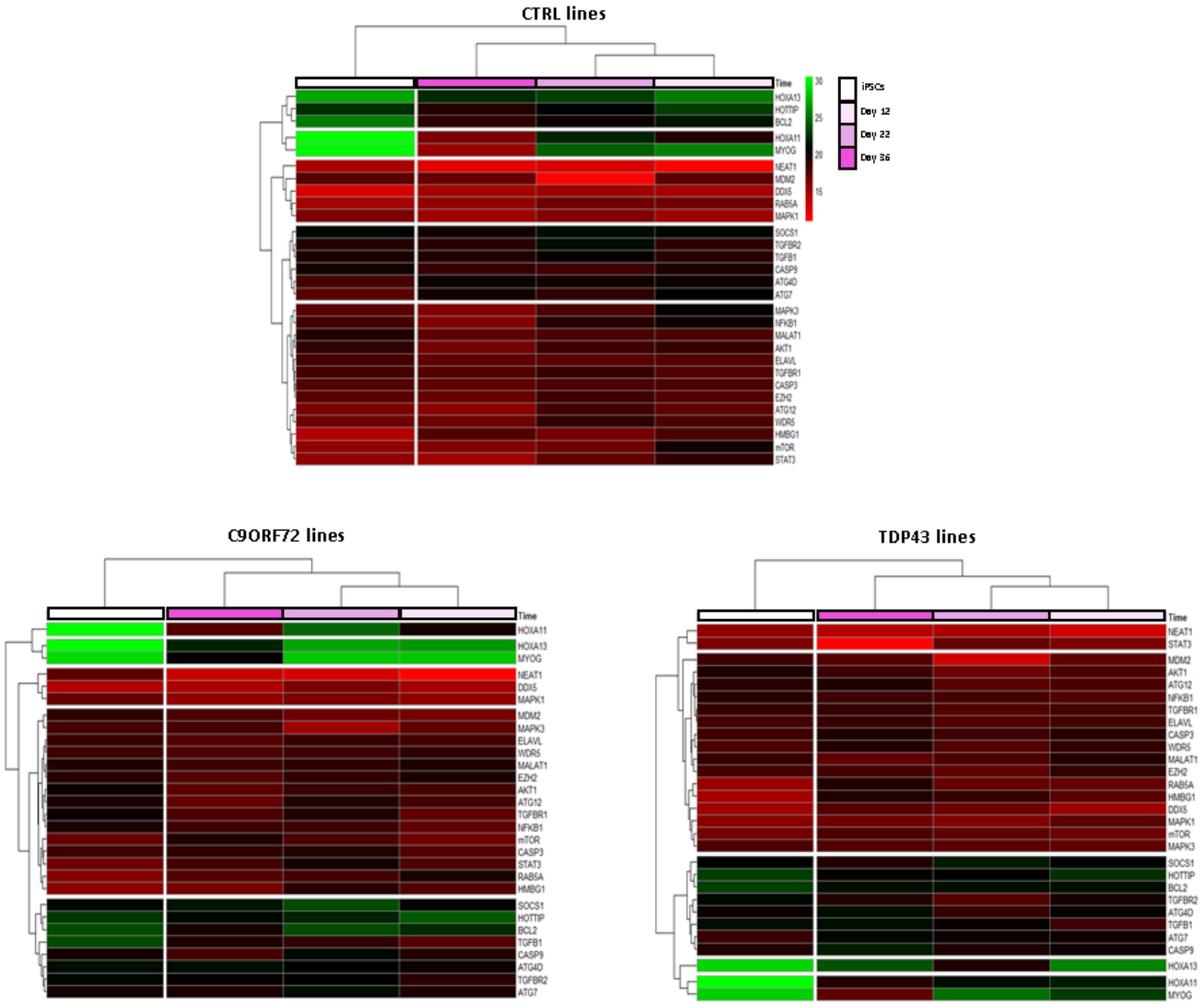

Figure 12. Hierarchical clustering heatmap of lncRNA and mRNA expressions. Heat map representation of lncRNAs and their target expression profile in control and ALS iPSCs and iPSC-derived muscle cells upon myogenic differentiation at days 12, 22, 36. Comparison of expression levels of differentially expressed lncRNAs and target genes, normalized against 18S endogenous control and calculated using the

ΔCt method, in control, C9ORF72 and TDP43 iPSCs, and muscle cells. In rainbow scale color, red indicates down-regulation and green indicates up-regulation of lncRNAs and their targets in control and ALS cells upon muscle differentiation (days 0, 12, 22, 36).

In particular, as shown by heat maps, C9ORF72 and TDP43 cell lines displayed a similar clusterisation of the mRNA targets, presenting two identical clusters, respectively composed of *HOXA11, HOXA13* and *MYOG* for one cluster, and *SOCS1, HOTTIP, BCL2, TGFB1, CASP9, ATG4D, TGFBR2* and *ATG7* for the other cluster. Moreover, C9ORF72 and TDP43 shared another mRNA target cluster, comprehending *AKT1, ATG12, CASP3, ELAVL, EZH2, HMBG1, MALAT1, MAPK3, MDM2, mTOR, NFKB1, RAB5A, TGFBR1* and *WDR5*. However, selected lncRNAs and mRNA targets showed different expression levels between C9ORF72 and TDP43, as shown in the heat maps. On the other hand, in CTRL cell lines *HOXA11, HOXA13* and *MYOG* still shape a cluster, but together with other molecules, as HOTTIP and BCL2. These data suggest that MALAT1, NEAT1, HOTTIP and mRNA targets, regulate a wide range of molecular and cellular pathways upon myogenic differentiation process in relation to ALS pathogenesis.

4 Discussion

ALS is a devastating neurodegenerative disease characterized by the progressive loss of upper and lower MNs [Kim G. et al, 2020]. Even though it is generally accepted that ALS is primarily caused by MN death, growing evidences have shown that the skeletal muscle has an active and crucial role in the disease onset and progression [Scaricamazza S. et al, 2021].

Notably, almost all phases of skeletal muscle development are regulated by lncRNAs, and specifically by MALAT1, NEAT1 and HOTTIP that are selectively enriched in muscle tissue and involved in myogenic processes [Butchart L.C. et al, 2016; Chen X. et al, 2017; Han X. et al, 2015; Hitachi K. et al, 2020; Jensen J.H. et al, 2012; Lai K.M. et al, 2015; Lehnert S.A. et al, 2007; Pradeepa M.M. et al, 2017; Sunwoo H. et al, 2009; Wang K.C. et al, 2011; Wang S. et al, 2019b; Watts R. et al, 2013; Zhang X. et al, 2017; Zheng L. et al, 2019].

In the present study, we investigated the expression of MALAT1, NEAT1 and HOTTIP lncRNAs, along with their putative target genes in iPSC-derived skeletal muscle cells from ALS patients and controls. Our aim was to explore the role of these lncRNAs in skeletal muscle development and function in the context of ALS disease, and hence their potential as therapeutic targets for counteracting the disease.

Firstly we demonstrated that iPSC-derived fibroblasts presenting the p.A382T mutation in TARDP gene or carrying the G_4C_2-repeat expansion in the C9ORF72 gene were able to differentiate into spontaneous contractile myotubes. According to the previous protocol published by Swartz and colleagues, iPSC-derived myotubes maintained the genetic background of ALS patients, thus representing an ideal new patient-specific *in vitro* model to study and reveal molecular and cellular disease mechanisms, or to develop novel drugs [Swartz E.W. et al, 2016].

Our molecular analyses showed a low expression of early, *T, PAX3, PAX7, MYOD, MYOG*, and late, *DES, MHC2, MHC3, MHC7*, myogenic markers in undifferentiated iPSCs. At day 12 of differentiation, we observed a higher expression of myogenic markers for mesodermal progenitor stage, including *T, PAX3, PAX7, MYOD* and *MYOG*, compared to other time points. These findings indicate that our *in vitro* model displayed the typical markers of early muscle differentiation, in line with previous studies [Borchin B. et al, 2013; Zammit P.S. et al, 2006]. At the final differentiation stage (day 36) we detected an increased expression of terminal myogenic differentiation markers, such as *DES* and *MHC2, MHC3* and *MHC7*, compared to the other time points (days 12, 22), in all cell lines.

Of note, we found an opposite expression trend of *MHC2* and *MHC7* at day 36, with a lower expression of *MHC2* and higher expression of *MHC7* in ALS spontaneous contractile myotubes. On the other hand, healthy control cell lines showed a higher expression of *MHC2* and a lower expression of *MHC7* at day 36. These findings suggest that fast–twitching cells, normally regulated by the fast myosin isoform *MHC2*, may be defective in ALS lines. This phenomenon is compensated by the action of slow-twitch myofibers, generated by the expression of *MHC7*. Notably, the skeletal muscle is highly plastic in response to

environmental and physiological changes. Indeed, myofibers can change from fast-twitch to slow-twitch type to adapt to different events or stimuli, a phenomenon called fiber-type switching. Fiber-type switching is observed as a consequence of exercise and it improve muscle fatigue resistance. It is also a preventive mechanism to counteract myodegeneration, as observed in ALS patients and mice Hegedus J. et al, 2008; Peggion C. et al, 2017; Telerman-Toppet N. and Coërs C. 1978]. Our results strength the potential of our iPSC model as an *in vitro* model of ALS patient-specific pathogenic events related to myofiber changes from fast-twitch to slow-twitch type, as observed by our molecular studies.

It is generally accepted that an increased proliferative potential of $PAX7^+$, $MYOD^+$ and $MYOG^+$ SC, represents a regenerative mechanism to counteract muscle atrophy in the skeletal muscle of ALS patients [Pradat P.F. et al, 2011; Scaramozza A. et al, 2014]. Our molecular analysis unequivocally demonstrates that *PAX7, MYOD* and *MYOG* expression increased at the final stage of myogenic differentiation, and this increase was more evident in TDP43 compared to C9ORF72 cells. The overall findings highlight that myogenic regulatory markers are implicated in the pathogenesis of ALS in the skeletal muscle differentiation context.

Results from our iPSC model were again in line with data obtained from ALS patients, since we observed *in vitro* evident molecular myotube changes. In fact, muscle abnormalities associated with ALS include changes in the expression of mitochondrial genes and a higher susceptibility to oxidative stress, as observed in ALS skeletal muscle [Loeffler J.P. et al, 2016]. Moreover, C9ORF72-ALS myocytes display a high expression and aggregation of TDP43, strongly indicating that skeletal muscle cells experience pathological changes caused by C9ORF72 mutation [Lynch E. M. et al, 2021]. In line with these

evidences, our molecular analyses demonstrated a differential expression pattern of myogenic markers, in ALS cell lines, particularly in C9ORF72 compared to controls. We hypothesize that the cytotoxicity induced by TDP43 aggregates and the alteration of RNA processing caused by C9ORF72 repeat expansion could induce a change in the amount and timing of expression of myogenic markers, in turn causing an intrinsic delay in the correct development and functionality of the skeletal muscle. These findings revealed a transcriptomic pattern specifically associated with muscle differentiation and that is potentially implicated in ALS.

Data from our *in vitro* model thus provide the foundation for further functional studies on cellular and molecular pathological alterations in ALS skeletal muscle, in order to discover new therapeutic targets against this devastating disease.

The precise cause of muscle atrophy in ALS is not well established. Thus, a greater knowledge of the different molecular pathways underlying the 'motor nerve ending–muscle fiber' cross talk could open new therapeutic perspectives [Giagnorio E. et al, 2021]. Our findings identified MALAT1, NEAT1 and HOTTIP lncRNAs as key molecules involved in myogenic differentiation, in relation to ALS pathogenesis. Indeed, our results showed an increased expression of NEAT1 upon muscle differentiation, specifically in the first stages of differentiation, which suggested a role for this molecule in the myogenesis, at the myoblast state as described by Wang and colleagues [Wang S. et al, 2019b]. The expression of HOTTIP was higher in iPSC-derived skeletal muscle cells compared to early mesodermal progenitor cells and iPSCs, supporting the idea that this lncRNA might have a crucial role in the development of the skeletal muscle. This finding is in line with a previous study demonstrating that HOTTIP regulates skeletal muscle development by targeting WDR5 and MLL protein complex [Wang K.C.

et al, 2011]. Interestingly, HOTTIP expression was higher in ALS lines compared to control cells, suggesting that dysregulation of this lncRNA might be dependent on ALS mutations, as observed for Myolinc lncRNA that binds TDP43, thus controlling the expression of myogenic regulatory factors [Militello G. et al, 2018]. Even though our results should be confirmed and extended in future studies, due to the limited sample size, they highlight the potential role of HOTTIP and NEAT1 as players in muscle dysregulation in ALS. Thus, they could represent possible targets for new therapeutic strategies to modulate muscle function and improve disease symptoms.

By applying hierarchical clustering to the expression of lncRNAs and their putative mRNA targets, we revealed specific lncRNA/mRNA clusters associated with a distinct myogenic differentiation stage in ALS and control cells, suggesting that an altered expression of these molecules might contribute to disease pathogenesis. In particular, we identified a cluster specific for C9ORF72 and TDP43 lines, which included *HOXA11, HOXA13* and *MYOG*.

HOXA genes are responsible for the embryonic development and they were shown to play a key role in the development of skeletal muscle. Specifically, *HOXA11* and *HOXA13* were found to participate in limb muscle development by reducing *MYOD* expression. Combined with *MYOG* hyper-expression, it is crucial for the early myogenic process at the myoblast stage [Yamamoto M. et al, 2003]. Our molecular analysis showed a higher expression of *HOXA11, HOXA13* and *MYOG* in C9ORF72 and TDP43 compared to control lines, at the iPSC stage, when the cell showed a pluripotent capacity typical of undifferentiated cells [Takahashi K. et al, 2007]. This result suggests the presence of intrinsic molecular alterations in iPSCs, likely depending on ALS related mutations, that cause an impairment of the myogenic process and an altered cellular muscle morphology.

It was already demonstrated the involvement of SOCS1 in inhibiting the skeletal muscle differentiation through a negative feedback regulation of IGF-I receptor-mediated signalling in myoblasts [Inaba M. et al, 2005]. In line with this previous study, C9ORF72 mesodermal progenitors at day 12 showed an increased expression of SOCS1 compared to TDP43 and control cells. Our data support the idea that C9ORF72 repeat expansion may negatively influence myogenic differentiation processes, determining an altered molecular muscle signature closely related to the altered muscle morphology.

Our overall data identified MALAT1, NEAT and HOTTIP as lncRNAs implicated in the differentiation program from iPSCs to iPSC-derived myotubes, and as molecules whose altered expression might significantly affect signalling pathways relevant for ALS-related pathogenic processes. Of interest, hierarchical clustering analysis of lncRNA expression levels in the axis "iPSCs-iPSC derived myotubes" revealed specific clusters characterizing the stages of the myogenic differentiation in ALS and control cells, suggesting that altered expression of these molecules might contribute to the disease pathogenesis in relation to muscle atrophy.

Our findings about the dysregulation of MALAT1, NEAT and HOTTIP, and their target genes, in the iPSC-derived ALS *in vitro* model provide new insights into ALS molecular basis, pointing out the possibility that altered muscle differentiation processes, depending on these lncRNAs, could eventually lead to an altered availability of muscle mass and function in the disease. Further studies in genetically defined, or not defined, ALS patients, and in other MNDs, could help to deeply understand the synergistic effect of MALAT1, NEAT and HOTTIP in disease onset and/or progression, towards future development of patient-specific lncRNA-based therapeutic strategies for ALS and other MNDs.

References

Amin A, Perera ND, Beart PM, Turner BJ, Shabanpoor F. Amyotrophic Lateral Sclerosis and Autophagy: Dysfunction and Therapeutic Targeting. Cells. 2020 Nov 4;9(11):2413. doi: 10.3390/cells9112413. PMID: 33158177; PMCID: PMC7694295.

Anderson DM, Anderson KM, Chang CL, Makarewich CA, Nelson BR, McAnally JR, Kasaragod P, Shelton JM, Liou J, Bassel-Duby R, Olson EN. A micropeptide encoded by a putative long noncoding RNA regulates muscle performance. Cell. 2015 Feb 12;160(4):595-606. doi: 10.1016/j.cell.2015.01.009. Epub 2015 Jan 29. PMID: 25640239; PMCID: PMC4356254.

Ballarino M, Cazzella V, D'Andrea D, et al. Novel long noncoding RNAs (lncRNAs) in myogenesis: a miR-31 overlapping lncRNA transcript controls myoblast differentiation [published correction appears in Mol Cell Biol. 2018 May 29;38(12):]. Mol Cell Biol. 2015;35(4):728-736. doi:10.1128/MCB.01394-14.

Bardelli D, Sassone F, Colombrita C, Volpe C, Gumina V, Peverelli S, Catusi I, Ratti A, Silani V, Bossolasco P. Reprogramming fibroblasts and peripheral blood cells from a C9ORF72 patient: A proof-of-principle study. J Cell Mol Med. 2020 Apr;24(7):4051-4060. doi: 10.1111/jcmm.15048. Epub 2020 Mar 3. PMID: 32125773; PMCID: PMC7171411.

Beltran M, Puig I, Peña C, García JM, Alvarez AB, Peña R, Bonilla F, de Herreros AG. A natural antisense transcript regulates Zeb2/Sip1 gene expression during Snail1-induced epithelial-mesenchymal transition. Genes Dev. 2008 Mar 15;22(6):756-69. doi: 10.1101/gad.455708. PMID: 18347095; PMCID: PMC2275429.

Bernard D, Prasanth KV, Tripathi V, Colasse S, Nakamura T, Xuan Z, Zhang MQ, Sedel F, Jourdren L, Coulpier F, Triller A, Spector DL, Bessis A. A long nuclear-retained non-coding RNA regulates synaptogenesis by modulating gene expression. EMBO J. 2010 Sep 15;29(18):3082-93. doi:

10.1038/emboj.2010.199. Epub 2010 Aug 20. PMID: 20729808; PMCID: PMC2944070.

Blokhuis AM, Groen EJ, Koppers M, van den Berg LH, Pasterkamp RJ. Protein aggregation in amyotrophic lateral sclerosis. Acta Neuropathol. 2013 Jun;125(6):777-94. doi: 10.1007/s00401-013-1125-6. Epub 2013 May 15. PMID: 23673820; PMCID: PMC3661910.

Bond CS, Fox AH. Paraspeckles: nuclear bodies built on long noncoding RNA. J Cell Biol. 2009 Sep 7;186(5):637-44. doi: 10.1083/jcb.200906113. Epub 2009 Aug 31. PMID: 19720872; PMCID: PMC2742191.

Borchin B, Chen J, Barberi T. Derivation and FACS-mediated purification of PAX3+/PAX7+ skeletal muscle precursors from human pluripotent stem cells. Stem Cell Reports. 2013 Nov 27;1(6):620-31. doi: 10.1016/j.stemcr.2013.10.007. PMID: 24371814; PMCID: PMC3871395.

Boylan K. Familial Amyotrophic Lateral Sclerosis. Neurol Clin. 2015 Nov;33(4):807-30. doi: 10.1016/j.ncl.2015.07.001. Epub 2015 Sep 8. PMID: 26515623; PMCID: PMC4670044.

Brown, R. H., & Al-Chalabi, A. (2017). Amyotrophic Lateral Sclerosis. The New England journal of medicine, 377(2), 162–172.

Buratti E, Baralle FE. Multiple roles of TDP-43 in gene expression, splicing regulation, and human disease. Front Biosci. 2008 Jan 1;13:867-78. doi: 10.2741/2727. PMID: 17981595.

Butchart LC, Fox A, Shavlakadze T, Grounds MD. The long and short of non-coding RNAs during post-natal growth and differentiation of skeletal muscles: Focus on lncRNA and miRNAs. Differentiation. 2016 Dec;92(5):237-248. doi: 10.1016/j.diff.2016.05.003. Epub 2016 Jun 10. PMID: 27292314.

Butti Z, Patten SA. RNA Dysregulation in Amyotrophic Lateral Sclerosis. Front Genet. 2019 Jan 22;9:712. doi: 10.3389/fgene.2018.00712. PMID: 30723494; PMCID: PMC6349704.

Caretti G, Schiltz RL, Dilworth FJ, Di Padova M, Zhao P, Ogryzko V, Fuller-Pace FV, Hoffman EP, Tapscott SJ, Sartorelli V. The RNA helicases p68/p72 and the noncoding RNA SRA are coregulators of MyoD and skeletal muscle differentiation. Dev Cell. 2006 Oct;11(4):547-60. doi: 10.1016/j.devcel.2006.08.003. PMID: 17011493.

Cazzato D, Castori M, Lombardi R, Caravello F, Bella ED, Petrucci A, Grammatico P, Dordoni C, Colombi M, Lauria G. Small fiber neuropathy is a common feature of Ehlers-Danlos syndromes. Neurology. 2016 Jul 12;87(2):155-9. doi: 10.1212/WNL.0000000000002847. Epub 2016 Jun 15. PMID: 27306637; PMCID: PMC4940063.

Cerase A, Smeets D, Tang YA, Gdula M, Kraus F, Spivakov M, Moindrot B, Leleu M, Tattermusch A, Demmerle J, Nesterova TB, Green C, Otte AP,

Schermelleh L, Brockdorff N. Spatial separation of Xist RNA and polycomb proteins revealed by superresolution microscopy. Proc Natl Acad Sci U S A. 2014 Feb 11;111(6):2235-40. doi: 10.1073/pnas.1312951111. Epub 2014 Jan 27. PMID: 24469834; PMCID: PMC3926064.

Cesana M, Cacchiarelli D, Legnini I, Santini T, Sthandier O, Chinappi M, Tramontano A, Bozzoni I. A long noncoding RNA controls muscle differentiation by functioning as a competing endogenous RNA. Cell. 2011 Oct 14;147(2):358-69. doi: 10.1016/j.cell.2011.09.028. Erratum in: Cell. 2011 Nov 11;147(4):947. PMID: 22000014; PMCID: PMC3234495.

Chal J, Pourquié O. Making muscle: skeletal myogenesis in vivo and in vitro. Development. 2017 Jun 15;144(12):2104-2122. doi: 10.1242/dev.151035. PMID: 28634270.

Chen X, He L, Zhao Y, Li Y, Zhang S, Sun K, So K, Chen F, Zhou L, Lu L, Wang L, Zhu X, Bao X, Esteban MA, Nakagawa S, Prasanth KV, Wu Z, Sun H, Wang H. MALAT1 regulates myogenic differentiation and muscle regeneration through modulating MyoD transcriptional activity. Cell Discov. 2017 Mar 14;3:17002. doi: 10.1038/celldisc.2017.2. PMID: 28326190; PMCID: PMC5348715.

Chiò A, Logroscino G, Hardiman O, Swingler R, Mitchell D, Beghi E, Traynor BG; Eurals Consortium. Prognostic factors in ALS: A critical review. Amyotroph Lateral Scler. 2009 Oct-Dec;10(5-6):310-23. doi: 10.3109/17482960802566824. PMID: 19922118; PMCID: PMC3515205.

Cretoiu D, Pavelescu L, Duica F, Radu M, Suciu N, Cretoiu SM. Myofibers. Adv Exp Med Biol. 2018;1088:23-46. doi: 10.1007/978-981-13-1435-3_2. PMID: 30390246.

Dalla Bella E, Bersano E, Antonini G, Borghero G, Capasso M, Caponnetto C, Chiò A, Corbo M, Filosto M, Giannini F, Spataro R, Lunetta C, Mandrioli J, Messina S, Monsurrò MR, Mora G, Riva N, Rizzi R, Siciliano G, Silani V, Simone I, Sorarù G, Tugnoli V, Verriello L, Volanti P, Furlan R, Nolan JM, Abgueguen E, Tramacere I, Lauria G. The unfolded protein response in amyotrophic later sclerosis: results of a phase 2 trial. Brain. 2021 Oct 22;144(9):2635-2647. doi: 10.1093/brain/awab167. PMID: 33905493; PMCID: PMC8557337.

DeJesus-Hernandez M, Mackenzie IR, Boeve BF, Boxer AL, Baker M, Rutherford NJ, Nicholson AM, Finch NA, Flynn H, Adamson J, Kouri N, Wojtas A, Sengdy P, Hsiung GY, Karydas A, Seeley WW, Josephs KA, Coppola G, Geschwind DH, Wszolek ZK, Feldman H, Knopman DS, Petersen RC, Miller BL, Dickson DW, Boylan KB, Graff-Radford NR, Rademakers R. Expanded GGGGCC hexanucleotide repeat in noncoding region of C9ORF72 causes chromosome 9p-linked FTD and ALS. Neuron. 2011 Oct 20;72(2):245-56. doi: 10.1016/j.neuron.2011.09.011. Epub 2011 Sep 21. PMID: 21944778; PMCID: PMC3202986.

Di Pietro L, Baranzini M, Berardinelli MG, Lattanzi W, Monforte M, Tasca G, Conte A, Logroscino G, Michetti F, Ricci E, Sabatelli M, Bernardini C. Potential therapeutic targets for ALS: MIR206, MIR208b and MIR499 are modulated during disease progression in the skeletal muscle of patients. Sci Rep. 2017 Aug 25;7(1):9538. doi: 10.1038/s41598-017-10161-z. PMID: 28842714; PMCID: PMC5573384.

Dill TL, Carroll A, Pinheiro A, Gao J, Naya FJ. The long noncoding RNA Meg3 regulates myoblast plasticity and muscle regeneration through epithelial-mesenchymal transition. Development. 2021 Jan 15;148(2):dev194027. doi: 10.1242/dev.194027. PMID: 33298462.

Dimartino D, Colantoni A, Ballarino M, Martone J, Mariani D, Danner J, Bruckmann A, Meister G, Morlando M, Bozzoni I. The Long Non-coding RNA lnc-31 Interacts with Rock1 mRNA and Mediates Its YB-1-Dependent Translation. Cell Rep. 2018 Apr 17;23(3):733-740. doi: 10.1016/j.celrep.2018.03.101. PMID: 29669280; PMCID: PMC5917449.

Donnelly CJ, Zhang PW, Pham JT, Haeusler AR, Mistry NA, Vidensky S, Daley EL, Poth EM, Hoover B, Fines DM, Maragakis N, Tienari PJ, Petrucelli L, Traynor BJ, Wang J, Rigo F, Bennett CF, Blackshaw S, Sattler R, Rothstein JD. RNA toxicity from the ALS/FTD C9ORF72 expansion is mitigated by antisense intervention. Neuron. 2013 Oct 16;80(2):415-28. doi: 10.1016/j.neuron.2013.10.015. Erratum in: Neuron. 2013 Nov 20;80(4):1102. Heusler, Aaron R [corrected to Haeusler, Aaron R]. PMID: 24139042; PMCID: PMC4098943.

Du J, Zhang P, Zhao X, He J, Xu Y, Zou Q, Luo J, Shen L, Gu H, Tang Q, Li M, Jiang Y, Tang G, Bai L, Li X, Wang J, Zhang S, Zhu L. MicroRNA-351-5p mediates skeletal myogenesis by directly targeting lactamase-β and is regulated by lnc-mg. FASEB J. 2019 Feb;33(2):1911-1926. doi: 10.1096/fj.201701394RRR. Epub 2018 Sep 14. PMID: 30216112.

d'Ydewalle C, Ramos DM, Pyles NJ, Ng SY, Gorz M, Pilato CM, Ling K, Kong L, Ward AJ, Rubin LL, Rigo F, Bennett CF, Sumner CJ. The Antisense Transcript SMN-AS1 Regulates SMN Expression and Is a Novel Therapeutic Target for Spinal Muscular Atrophy. Neuron. 2017 Jan 4;93(1):66-79. doi: 10.1016/j.neuron.2016.11.033. Epub 2016 Dec 22. PMID: 28017471; PMCID: PMC5223741.

Echaniz-Laguna A, Zoll J, Ponsot E, N'guessan B, Tranchant C, Loeffler JP, Lampert E. Muscular mitochondrial function in amyotrophic lateral sclerosis is progressively altered as the disease develops: a temporal study in man. Exp Neurol. 2006 Mar;198(1):25-30. doi: 10.1016/j.expneurol.2005.07.020. Epub 2005 Aug 29. PMID: 16126198.

Elden AC, Kim HJ, Hart MP, Chen-Plotkin AS, Johnson BS, Fang X, Armakola M, Geser F, Greene R, Lu MM, Padmanabhan A, Clay-Falcone D, McCluskey L, Elman L, Juhr D, Gruber PJ, Rüb U, Auburger G, Trojanowski JQ, Lee VM, Van Deerlin VM, Bonini NM, Gitler AD. Ataxin-2 intermediate-

length polyglutamine expansions are associated with increased risk for ALS. Nature. 2010 Aug 26;466(7310):1069-75. doi: 10.1038/nature09320. PMID: 20740007; PMCID: PMC2965417.

Fiorenzano A, Pascale E, Patriarca EJ, Minchiotti G, Fico A. LncRNAs and PRC2: Coupled Partners in Embryonic Stem Cells. Epigenomes. 2019 Aug 6;3(3):14. doi: 10.3390/epigenomes3030014. PMID: 34968226; PMCID: PMC8594682.

Francaux M, Deldicque L. Exercise and the control of muscle mass in human. Pflugers Arch. 2019 Mar;471(3):397-411. doi: 10.1007/s00424-018-2217-x. Epub 2018 Oct 11. PMID: 30310991.

Frank S, Ahuja G, Bartsch D, Russ N, Yao W, Kuo JC, Derks JP, Akhade VS, Kargapolova Y, Georgomanolis T, Messling JE, Gramm M, Brant L, Rehimi R, Vargas NE, Kuroczik A, Yang TP, Sahito RGA, Franzen J, Hescheler J, Sachinidis A, Peifer M, Rada-Iglesias A, Kanduri M, Costa IG, Kanduri C, Papantonis A, Kurian L. yylncT Defines a Class of Divergently Transcribed lncRNAs and Safeguards the T-mediated Mesodermal Commitment of Human PSCs. Cell Stem Cell. 2019 Feb 7;24(2):318-327.e8. doi: 10.1016/j.stem.2018.11.005. Epub 2018 Dec 13. PMID: 30554961.

Fujimori K, Ishikawa M, Otomo A, Atsuta N, Nakamura R, Akiyama T, Hadano S, Aoki M, Saya H, Sobue G, Okano H. Modeling sporadic ALS in iPSC-derived motor neurons identifies a potential therapeutic agent. Nat Med. 2018 Oct;24(10):1579-1589. doi: 10.1038/s41591-018-0140-5. Epub 2018 Aug 20. PMID: 30127392.

Gagliardi S, Pandini C, Garofalo M, Bordoni M, Pansarasa O, Cereda C. Long non coding RNAs and ALS: Still much to do. Noncoding RNA Res. 2018 Nov 15;3(4):226-231. doi: 10.1016/j.ncrna.2018.11.004. PMID: 30533570; PMCID: PMC6260474.

Gendron TF, Bieniek KF, Zhang YJ, Jansen-West K, Ash PE, Caulfield T, Daughrity L, Dunmore JH, Castanedes-Casey M, Chew J, Cosio DM, van Blitterswijk M, Lee WC, Rademakers R, Boylan KB, Dickson DW, Petrucelli L. Antisense transcripts of the expanded C9ORF72 hexanucleotide repeat form nuclear RNA foci and undergo repeat-associated non-ATG translation in c9FTD/ALS. Acta Neuropathol. 2013 Dec;126(6):829-44. doi: 10.1007/s00401-013-1192-8. Epub 2013 Oct 16. PMID: 24129584; PMCID: PMC3830741.

Gene Ontology Consortium, Blake JA, Dolan M, Drabkin H, Hill DP, Li N, Sitnikov D, Bridges S, Burgess S, Buza T, McCarthy F, Peddinti D, Pillai L, Carbon S, Dietze H, Ireland A, Lewis SE, Mungall CJ, Gaudet P, Chrisholm RL, Fey P, Kibbe WA, Basu S, Siegele DA, McIntosh BK, Renfro DP, Zweifel AE, Hu JC, Brown NH, Tweedie S, Alam-Faruque Y, Apweiler R, Auchinchloss A, Axelsen K, Bely B, Blatter M-, Bonilla C, Bouguerleret L, Boutet E, Breuza L, Bridge A, Chan WM, Chavali G, Coudert E, Dimmer E, Estreicher A, Famiglietti L, Feuermann M, Gos A, Gruaz-Gumowski N, Hieta R, Hinz C, Hulo C, Huntley R, James J, Jungo F, Keller G, Laiho K, Legge D,

Lemercier P, Lieberherr D, Magrane M, Martin MJ, Masson P, Mutowo-Muellenet P, O'Donovan C, Pedruzzi I, Pichler K, Poggioli D, Porras Millán P, Poux S, Rivoire C, Roechert B, Sawford T, Schneider M, Stutz A, Sundaram S, Tognolli M, Xenarios I, Foulgar R, Lomax J, Roncaglia P, Khodiyar VK, Lovering RC, Talmud PJ, Chibucos M, Giglio MG, Chang H-, Hunter S, McAnulla C, Mitchell A, Sangrador A, Stephan R, Harris MA, Oliver SG, Rutherford K, Wood V, Bahler J, Lock A, Kersey PJ, McDowall DM, Staines DM, Dwinell M, Shimoyama M, Laulederkind S, Hayman T, Wang S-, Petri V, Lowry T, D'Eustachio P, Matthews L, Balakrishnan R, Binkley G, Cherry JM, Costanzo MC, Dwight SS, Engel SR, Fisk DG, Hitz BC, Hong EL, Karra K, Miyasato SR, Nash RS, Park J, Skrzypek MS, Weng S, Wong ED, Berardini TZ, Huala E, Mi H, Thomas PD, Chan J, Kishore R, Sternberg P, Van Auken K, Howe D, Westerfield M. Gene Ontology annotations and resources. Nucleic Acids Res. 2013 Jan;41(Database issue):D530-5. doi: 10.1093/nar/gks1050. Epub 2012 Nov 17. PMID: 23161678; PMCID: PMC3531070.

Giagnorio E, Malacarne C, Mantegazza R, Bonanno S, Marcuzzo S. MyomiRs and their multifaceted regulatory roles in muscle homeostasis and amyotrophic lateral sclerosis. J Cell Sci. 2021 Jun 15;134(12):jcs258349. doi: 10.1242/jcs.258349. Epub 2021 Jun 17. PMID: 34137441.

Gong C, Li Z, Ramanujan K, Clay I, Zhang Y, Lemire-Brachat S, Glass DJ. A long non-coding RNA, LncMyoD, regulates skeletal muscle differentiation by blocking IMP2-mediated mRNA translation. Dev Cell. 2015 Jul 27;34(2):181-91. doi: 10.1016/j.devcel.2015.05.009. Epub 2015 Jul 2. PMID: 26143994.

Gurney ME, Pu H, Chiu AY, Dal Canto MC, Polchow CY, Alexander DD, Caliendo J, Hentati A, Kwon YW, Deng HX, et al. Motor neuron degeneration in mice that express a human Cu,Zn superoxide dismutase mutation. Science. 1994 Jun 17;264(5166):1772-5. doi: 10.1126/science.8209258. Erratum in: Science 1995 Jul 14;269(5221):149. PMID: 8209258.

Guttman M, Rinn JL. Modular regulatory principles of large non-coding RNAs. Nature. 2012 Feb 15;482(7385):339-46. doi: 10.1038/nature10887. PMID: 22337053; PMCID: PMC4197003.

Halon-Golabek M, Borkowska A, Kaczor JJ, Ziolkowski W, Flis DJ, Knap N, Kasperuk K, Antosiewicz J. hmSOD1 gene mutation-induced disturbance in iron metabolism is mediated by impairment of Akt signalling pathway. J Cachexia Sarcopenia Muscle. 2018 Jun;9(3):557-569. doi: 10.1002/jcsm.12283. Epub 2018 Jan 29. PMID: 29380557; PMCID: PMC5989766.

Han X, Yang F, Cao H, Liang Z. MALAT1 regulates serum response factor through miR-133 as a competing endogenous RNA in myogenesis. FASEB J. 2015 Jul;29(7):3054-64. doi: 10.1096/fj.14-259952. Epub 2015 Apr 13. PMID: 25868726.

Hardiman O, Al-Chalabi A, Chio A, Corr EM, Logroscino G, Robberecht W, Shaw PJ, Simmons Z, van den Berg LH. Amyotrophic lateral sclerosis. Nat Rev Dis Primers. 2017 Oct 5;3:17071. doi: 10.1038/nrdp.2017.71. Erratum in: Nat Rev Dis Primers. 2017 Oct 20;3:17085. PMID: 28980624.

He C, Sidoli S, Warneford-Thomson R, Tatomer DC, Wilusz JE, Garcia BA, Bonasio R. High-Resolution Mapping of RNA-Binding Regions in the Nuclear Proteome of Embryonic Stem Cells. Mol Cell. 2016 Oct 20;64(2):416-430. doi: 10.1016/j.molcel.2016.09.034. PMID: 27768875; PMCID: PMC5222606.

Hegedus J, Putman CT, Tyreman N, Gordon T. Preferential motor unit loss in the SOD1 G93A transgenic mouse model of amyotrophic lateral sclerosis. J Physiol. 2008 Jul 15;586(14):3337-51. doi: 10.1113/jphysiol.2007.149286. Epub 2008 May 8. PMID: 18467368; PMCID: PMC2538809.

Hitachi K, Nakatani M, Tsuchida K. Long Non-Coding RNA Myoparr Regulates GDF5 Expression in Denervated Mouse Skeletal Muscle. Noncoding RNA. 2019 Apr 8;5(2):33. doi: 10.3390/ncrna5020033. PMID: 30965639; PMCID: PMC6631233.

Hitachi K, Nakatani M, Funasaki S, Hijikata I, Maekawa M, Honda M, Tsuchida K. Expression Levels of Long Non-Coding RNAs Change in Models of Altered Muscle Activity and Muscle Mass. Int J Mol Sci. 2020 Feb 27;21(5):1628. doi: 10.3390/ijms21051628. PMID: 32120896; PMCID: PMC7084395.

Hoekstra SD, Stringer S, Heine VM, Posthuma D. Genetically-Informed Patient Selection for iPSC Studies of Complex Diseases May Aid in Reducing Cellular Heterogeneity. Front Cell Neurosci. 2017 Jun 13;11:164. doi: 10.3389/fncel.2017.00164. PMID: 28659762; PMCID: PMC5468546.

Hoppeler H. Molecular networks in skeletal muscle plasticity. J Exp Biol. 2016 Jan;219(Pt 2):205-13. doi: 10.1242/jeb.128207. PMID: 26792332.

http://123.59.132.21/lncrna2target/search.jsp

http://biocc.hrbmu.edu.cn/LncTarD/JumpToAdvancedSearch#tableDivHead

Huynh W, Ahmed R, Mahoney CJ, Nguyen C, Tu S, Caga J, Loh P, Lin CS, Kiernan MC. The impact of cognitive and behavioral impairment in amyotrophic lateral sclerosis. Expert Rev Neurother. 2020 Mar;20(3):281-293. doi: 10.1080/14737175.2020.1727740. Epub 2020 Feb 14. PMID: 32031423.

Ilieva H, Polymenidou M, Cleveland DW. Non-cell autonomous toxicity in neurodegenerative disorders: ALS and beyond. J Cell Biol. 2009 Dec 14;187(6):761-72. doi: 10.1083/jcb.200908164. PMID: 19951898; PMCID: PMC2806318.

Inaba M, Saito H, Fujimoto M, Sumitani S, Ohkawara T, Tanaka T, Kouhara H, Kasayama S, Kawase I, Kishimoto T, Naka T. Suppressor of cytokine signaling 1 suppresses muscle differentiation through modulation of IGF-I

receptor signal transduction. Biochem Biophys Res Commun. 2005 Mar 25;328(4):953-61. doi: 10.1016/j.bbrc.2005.01.046. PMID: 15707970.

Ishikawa K, Yoshida K, Kanie K, Omori K, Kato R. Morphology-Based Analysis of Myoblasts for Prediction of Myotube Formation. SLAS Discov. 2019 Jan;24(1):47-56. doi: 10.1177/2472555218793374. Epub 2018 Aug 13. PMID: 30102873.

Jaarsma D, Teuling E, Haasdijk ED, De Zeeuw CI, Hoogenraad CC. Neuron-specific expression of mutant superoxide dismutase is sufficient to induce amyotrophic lateral sclerosis in transgenic mice. J Neurosci. 2008 Feb 27;28(9):2075-88. doi: 10.1523/JNEUROSCI.5258-07.2008. PMID: 18305242; PMCID: PMC6671838.

Jensen JH, Conley LN, Hedegaard J, Nielsen M, Young JF, Oksbjerg N, Hornshøj H, Bendixen C, Thomsen B. Gene expression profiling of porcine skeletal muscle in the early recovery phase following acute physical activity. Exp Physiol. 2012 Jul;97(7):833-48. doi: 10.1113/expphysiol.2011.063727. Epub 2012 Feb 15. PMID: 22337866.

Ji P, Diederichs S, Wang W, Böing S, Metzger R, Schneider PM, Tidow N, Brandt B, Buerger H, Bulk E, Thomas M, Berdel WE, Serve H, Müller-Tidow C. MALAT-1, a novel noncoding RNA, and thymosin beta4 predict metastasis and survival in early-stage non-small cell lung cancer. Oncogene. 2003 Sep 11;22(39):8031-41. doi: 10.1038/sj.onc.1206928. PMID: 12970751.

Jin JJ, Lv W, Xia P, Xu ZY, Zheng AD, Wang XJ, Wang SS, Zeng R, Luo HM, Li GL, Zuo B. Long noncoding RNA SYISL regulates myogenesis by interacting with polycomb repressive complex 2. Proc Natl Acad Sci U S A. 2018 Oct 16;115(42):E9802-E9811. doi: 10.1073/pnas.1801471115. Epub 2018 Oct 2. PMID: 30279181; PMCID: PMC6196504.

Jiwlawat N, Lynch E, Jeffrey J, Van Dyke JM, Suzuki M. Current Progress and Challenges for Skeletal Muscle Differentiation from Human Pluripotent Stem Cells Using Transgene-Free Approaches. Stem Cells Int. 2018 Apr 11;2018:6241681. doi: 10.1155/2018/6241681. PMID: 29760730; PMCID: PMC5924987.

Kabashi E, Lin L, Tradewell ML, Dion PA, Bercier V, Bourgouin P, Rochefort D, Bel Hadj S, Durham HD, Vande Velde C, Rouleau GA, Drapeau P. Gain and loss of function of ALS-related mutations of TARDBP (TDP-43) cause motor deficits in vivo. Hum Mol Genet. 2010 Feb 15;19(4):671-83. doi: 10.1093/hmg/ddp534. Epub 2009 Dec 3. Erratum in: Hum Mol Genet. 2010 Aug 1;19(15):3102. PMID: 19959528.

Kanehisa M, Goto S. KEGG: kyoto encyclopedia of genes and genomes. Nucleic Acids Res. 2000 Jan 1;28(1):27-30. doi: 10.1093/nar/28.1.27. PMID: 10592173; PMCID: PMC102409.

Keihani S, Kluever V, Mandad S, Bansal V, Rahman R, Fritsch E, Gomes LC, Gärtner A, Kügler S, Urlaub H, Wren JD, Bonn S, Rizzoli SO, Fornasiero EF.

The long noncoding RNA neuroLNC regulates presynaptic activity by interacting with the neurodegeneration-associated protein TDP-43. Sci Adv. 2019 Dec 18;5(12):eaay2670. doi: 10.1126/sciadv.aay2670. PMID: 31897430; PMCID: PMC6920028.

Kim G, Gautier O, Tassoni-Tsuchida E, Ma XR, Gitler AD. ALS Genetics: Gains, Losses, and Implications for Future Therapies. Neuron. 2020 Dec 9;108(5):822-842. doi: 10.1016/j.neuron.2020.08.022. Epub 2020 Sep 14. PMID: 32931756; PMCID: PMC7736125.

Koishi K, Zhang M, McLennan IS, Harris AJ. MyoD protein accumulates in satellite cells and is neurally regulated in regenerating myotubes and skeletal muscle fibers. Dev Dyn. 1995 Mar;202(3):244-54. doi: 10.1002/aja.1002020304. PMID: 7780174.

Kook MG, Lee S, Shin N, Kong D, Kim DH, Kim MS, Kang HK, Choi SW, Kang KS. Repeated intramuscular transplantations of hUCB-MSCs improves motor function and survival in the SOD1 $G^{93}A$ mice through activation of AMPK. Sci Rep. 2020 Jan 31;10(1):1572. doi: 10.1038/s41598-020-58221-1. PMID: 32005848; PMCID: PMC6994691.

Kuida K, Haydar TF, Kuan CY, Gu Y, Taya C, Karasuyama H, Su MS, Rakic P, Flavell RA. Reduced apoptosis and cytochrome c-mediated caspase activation in mice lacking caspase 9. Cell. 1998 Aug 7;94(3):325-37. doi: 10.1016/s0092-8674(00)81476-2. PMID: 9708735.

Lagier-Tourenne C, Polymenidou M, Hutt KR, Vu AQ, Baughn M, Huelga SC, Clutario KM, Ling SC, Liang TY, Mazur C, Wancewicz E, Kim AS, Watt A, Freier S, Hicks GG, Donohue JP, Shiue L, Bennett CF, Ravits J, Cleveland DW, Yeo GW. Divergent roles of ALS-linked proteins FUS/TLS and TDP-43 intersect in processing long pre-mRNAs. Nat Neurosci. 2012 Nov;15(11):1488-97. doi: 10.1038/nn.3230. Epub 2012 Sep 30. PMID: 23023293; PMCID: PMC3586380.

Lai C, Xie C, Shim H, Chandran J, Howell BW, Cai H. Regulation of endosomal motility and degradation by amyotrophic lateral sclerosis 2/alsin. Mol Brain. 2009 Jul 24;2:23. doi: 10.1186/1756-6606-2-23. PMID: 19630956; PMCID: PMC2724476.

Lai KM, Gong G, Atanasio A, Rojas J, Quispe J, Posca J, White D, Huang M, Fedorova D, Grant C, Miloscio L, Droguett G, Poueymirou WT, Auerbach W, Yancopoulos GD, Frendewey D, Rinn J, Valenzuela DM. Diverse Phenotypes and Specific Transcription Patterns in Twenty Mouse Lines with Ablated LincRNAs. PLoS One. 2015 Apr 24;10(4):e0125522. doi: 10.1371/journal.pone.0125522. PMID: 25909911; PMCID: PMC4409293.

Lamb CA, Dooley HC, Tooze SA. Endocytosis and autophagy: Shared machinery for degradation. Bioessays. 2013 Jan;35(1):34-45. doi: 10.1002/bies.201200130. Epub 2012 Nov 13. PMID: 23147242.

Laneve P, Tollis P, Caffarelli E. RNA Deregulation in Amyotrophic Lateral Sclerosis: The Noncoding Perspective. Int J Mol Sci. 2021 Sep

24;22(19):10285. doi: 10.3390/ijms221910285. PMID: 34638636; PMCID: PMC8508793.

Le Gall L, Anakor E, Connolly O, Vijayakumar UG, Duddy WJ, Duguez S. Molecular and Cellular Mechanisms Affected in ALS. J Pers Med. 2020 Aug 25;10(3):101. doi: 10.3390/jpm10030101. PMID: 32854276; PMCID: PMC7564998.

Legnini I, Morlando M, Mangiavacchi A, Fatica A, Bozzoni I. A feedforward regulatory loop between HuR and the long noncoding RNA linc-MD1 controls early phases of myogenesis. Mol Cell. 2014 Feb 6;53(3):506-14. doi: 10.1016/j.molcel.2013.12.012. Epub 2014 Jan 16. PMID: 24440503; PMCID: PMC3919156.

Lehnert SA, Reverter A, Byrne KA, Wang Y, Nattrass GS, Hudson NJ, Greenwood PL. Gene expression studies of developing bovine longissimus muscle from two different beef cattle breeds. BMC Dev Biol. 2007 Aug 16;7:95. doi: 10.1186/1471-213X-7-95. PMID: 17697390; PMCID: PMC2031903.

Li J, Zhao W, Li Q, Huang Z, Shi G, Li C. Long Non-Coding RNA H19 Promotes Porcine Satellite Cell Differentiation by Interacting with TDP43. Genes (Basel). 2020 Feb 28;11(3):259. doi: 10.3390/genes11030259. PMID: 32121115; PMCID: PMC7140797.

Li M, Zhou X, Chen Y, Nie Y, Huang H, Chen H, Mo D. Not all the number of skeletal muscle fibers is determined prenatally. BMC Dev Biol. 2015 Nov 11;15:42. doi: 10.1186/s12861-015-0091-8. PMID: 26559169; PMCID: PMC4642764.

Li P, Ruan X, Yang L, Kiesewetter K, Zhao Y, Luo H, Chen Y, Gucek M, Zhu J, Cao H. A liver-enriched long non-coding RNA, lncLSTR, regulates systemic lipid metabolism in mice. Cell Metab. 2015 Mar 3;21(3):455-67. doi: 10.1016/j.cmet.2015.02.004. PMID: 25738460; PMCID: PMC4350020.

Li PP, Sun X, Xia G, Arbez N, Paul S, Zhu S, Peng HB, Ross CA, Koeppen AH, Margolis RL, Pulst SM, Ashizawa T, Rudnicki DD. ATXN2-AS, a gene antisense to ATXN2, is associated with spinocerebellar ataxia type 2 and amyotrophic lateral sclerosis. Ann Neurol. 2016 Oct;80(4):600-15. doi: 10.1002/ana.24761. PMID: 27531668; PMCID: PMC6555153.

Li Z, Cai B, Abdalla BA, Zhu X, Zheng M, Han P, Nie Q, Zhang X. LncIRS1 controls muscle atrophy via sponging miR-15 family to activate IGF1-PI3K/AKT pathway. J Cachexia Sarcopenia Muscle. 2019 Apr;10(2):391-410. doi: 10.1002/jcsm.12374. Epub 2019 Jan 30. PMID: 30701698; PMCID: PMC6463472.

Liang T, Zhou B, Shi L, Wang H, Chu Q, Xu F, Li Y, Chen R, Shen C, Schinckel AP. lncRNA AK017368 promotes proliferation and suppresses differentiation of myoblasts in skeletal muscle development by attenuating the

function of miR-30c. FASEB J. 2018 Jan;32(1):377-389. doi: 10.1096/fj.201700560RR. Epub 2017 Sep 13. PMID: 28904016.

Liu G, Coyne AN, Pei F, Vaughan S, Chaung M, Zarnescu DC, Buchan JR. Endocytosis regulates TDP-43 toxicity and turnover. Nat Commun. 2017 Dec 12;8(1):2092. doi: 10.1038/s41467-017-02017-x. PMID: 29233983; PMCID: PMC5727062.

Lobsiger CS, Cleveland DW. Glial cells as intrinsic components of non-cell-autonomous neurodegenerative disease. Nat Neurosci. 2007 Nov;10(11):1355-60. doi: 10.1038/nn1988. PMID: 17965655; PMCID: PMC3110080.

Loeffler JP, Picchiarelli G, Dupuis L, Gonzalez De Aguilar JL. The Role of Skeletal Muscle in Amyotrophic Lateral Sclerosis. Brain Pathol. 2016 Mar;26(2):227-36. doi: 10.1111/bpa.12350. PMID: 26780251; PMCID: PMC8029271.

Lynch EM, Robertson S, FitzGibbons C, Reilly M, Switalski C, Eckardt A, Tey SR, Hayakawa K, Suzuki M. Transcriptome analysis using patient iPSC-derived skeletal myocytes: Bet1L as a new molecule possibly linked to neuromuscular junction degeneration in ALS. Exp Neurol. 2021 Nov;345:113815. doi: 10.1016/j.expneurol.2021.113815. Epub 2021 Jul 24. PMID: 34310943; PMCID: PMC8429236.

Lynch EM, Semrad T, Belsito VS, FitzGibbons C, Reilly M, Hayakawa K, Suzuki M. *C9ORF72*-related cellular pathology in skeletal myocytes derived from ALS-patient induced pluripotent stem cells. Dis Model Mech. 2019 Aug 16;12(8):dmm039552. doi: 10.1242/dmm.039552. PMID: 31439573; PMCID: PMC6737948.

Malacarne C, Galbiati M, Giagnorio E, Cavalcante P, Salerno F, Andreetta F, Cagnoli C, Taiana M, Nizzardo M, Corti S, Pensato V, Venerando A, Gellera C, Fenu S, Pareyson D, Masson R, Maggi L, Dalla Bella E, Lauria G, Mantegazza R, Bernasconi P, Poletti A, Bonanno S, Marcuzzo S. Dysregulation of Muscle-Specific MicroRNAs as Common Pathogenic Feature Associated with Muscle Atrophy in ALS, SMA and SBMA: Evidence from Animal Models and Human Patients. Int J Mol Sci. 2021 May 26;22(11):5673. doi: 10.3390/ijms22115673. PMID: 34073630; PMCID: PMC8198536.

Mao YS, Zhang B, Spector DL. Biogenesis and function of nuclear bodies. Trends Genet. 2011 Aug;27(8):295-306. doi: 10.1016/j.tig.2011.05.006. Epub 2011 Jun 15. PMID: 21680045; PMCID: PMC3144265.

Marchese FP, Raimondi I, Huarte M. The multidimensional mechanisms of long noncoding RNA function. Genome Biol. 2017 Oct 31;18(1):206. doi: 10.1186/s13059-017-1348-2. PMID: 29084573; PMCID: PMC5663108.

Marcuzzo S, Zucca I, Mastropietro A, de Rosbo NK, Cavalcante P, Tartari S, Bonanno S, Preite L, Mantegazza R, Bernasconi P. Hind limb muscle atrophy precedes cerebral neuronal degeneration in G93A-SOD1 mouse model of

amyotrophic lateral sclerosis: a longitudinal MRI study. Exp Neurol. 2011 Sep;231(1):30-7. doi: 10.1016/j.expneurol.2011.05.007. Epub 2011 May 14. PMID: 21620832.

Marcuzzo S, Terragni B, Bonanno S, Isaia D, Cavalcante P, Cappelletti C, Ciusani E, Rizzo A, Regalia G, Yoshimura N, Ugolini GS, Rasponi M, Bechi G, Mantegazza M, Mantegazza R, Bernasconi P, Minati L. Hyperexcitability in Cultured Cortical Neuron Networks from the G93A-SOD1 Amyotrophic Lateral Sclerosis Model Mouse and its Molecular Correlates. Neuroscience. 2019 Sep 15;416:88-99. doi: 10.1016/j.neuroscience.2019.07.041. Epub 2019 Aug 7. PMID: 31400485.

Martin LJ, Wong M. Skeletal Muscle-Restricted Expression of Human SOD1 in Transgenic Mice Causes a Fatal ALS-Like Syndrome. Front Neurol. 2020 Dec 14;11:592851. doi: 10.3389/fneur.2020.592851. PMID: 33381076; PMCID: PMC7767933.

Mathews DH, Moss WN, Turner DH. Folding and finding RNA secondary structure. Cold Spring Harb Perspect Biol. 2010 Dec;2(12):a003665. doi: 10.1101/cshperspect.a003665. Epub 2010 Aug 4. PMID: 20685845; PMCID: PMC2982177.

Matsumoto A, Pasut A, Matsumoto M, Yamashita R, Fung J, Monteleone E, Saghatelian A, Menon P, Kiernan MC, Vucic S. Cortical hyperexcitability precedes lower motor neuron dysfunction in ALS. Clin Neurophysiol. 2015 Apr;126(4):803-9. doi: 10.1016/j.clinph.2014.04.023. Epub 2014 Aug 28. PMID: 25227219.

Menon P, Kiernan MC, Vucic S. Cortical hyperexcitability precedes lower motor neuron dysfunction in ALS. Clin Neurophysiol. 2015 Apr;126(4):803-9. doi: 10.1016/j.clinph.2014.04.023. Epub 2014 Aug 28. PMID: 25227219.

Mercer TR, Mattick JS. Structure and function of long noncoding RNAs in epigenetic regulation. Nat Struct Mol Biol. 2013 Mar;20(3):300-7. doi: 10.1038/nsmb.2480. PMID: 23463315.

Militello G, Hosen MR, Ponomareva Y, Gellert P, Weirick T, John D, Hindi SM, Mamchaoui K, Mouly V, Döring C, Zhang L, Nakamura M, Kumar A, Fukada SI, Dimmeler S, Uchida S. A novel long non-coding RNA Myolinc regulates myogenesis through TDP-43 and Filip1. J Mol Cell Biol. 2018 Apr 1;10(2):102-117. doi: 10.1093/jmcb/mjy025. PMID: 29618024; PMCID: PMC7191624.

Mòdol-Caballero G, Herrando-Grabulosa M, García-Lareu B, Solanes N, Verdés S, Osta R, Francos-Quijorna I, López-Vales R, Calvo AC, Bosch A, Navarro X. Gene therapy for overexpressing Neuregulin 1 type I in skeletal muscles promotes functional improvement in the SOD1^{G93A} ALS mice. Neurobiol Dis. 2020 Apr;137:104793. doi: 10.1016/j.nbd.2020.104793. Epub 2020 Feb 4. PMID: 32032731.

Moloney EB, de Winter F, Verhaagen J. ALS as a distal axonopathy: molecular mechanisms affecting neuromuscular junction stability in the presymptomatic stages of the disease. Front Neurosci. 2014 Aug 14;8:252. doi: 10.3389/fnins.2014.00252. PMID: 25177267; PMCID: PMC4132373.

Mondal T, Subhash S, Vaid R, Enroth S, Uday S, Reinius B, Mitra S, Mohammed A, James AR, Hoberg E, Moustakas A, Gyllensten U, Jones SJ, Gustafsson CM, Sims AH, Westerlund F, Gorab E, Kanduri C. MEG3 long noncoding RNA regulates the TGF-β pathway genes through formation of RNA-DNA triplex structures. Nat Commun. 2015 Jul 24;6:7743. doi: 10.1038/ncomms8743. Erratum in: Nat Commun. 2019 Nov 21;10(1):5290. PMID: 26205790; PMCID: PMC4525211.

Mori F, Tada M, Kon T, Miki Y, Tanji K, Kurotaki H, Tomiyama M, Ishihara T, Onodera O, Kakita A, Wakabayashi K. Phosphorylated TDP-43 aggregates in skeletal and cardiac muscle are a marker of myogenic degeneration in amyotrophic lateral sclerosis and various conditions. Acta Neuropathol Commun. 2019 Oct 28;7(1):165. doi: 10.1186/s40478-019-0824-1. PMID: 31661037; PMCID: PMC6816170.

Mori K, Arzberger T, Grässer FA, Gijselinck I, May S, Rentzsch K, Weng SM, Schludi MH, van der Zee J, Cruts M, Van Broeckhoven C, Kremmer E, Kretzschmar HA, Haass C, Edbauer D. Bidirectional transcripts of the expanded C9orf72 hexanucleotide repeat are translated into aggregating dipeptide repeat proteins. Acta Neuropathol. 2013 Dec;126(6):881-93. doi: 10.1007/s00401-013-1189-3. Epub 2013 Oct 17. PMID: 24132570.

Mueller AC, Cichewicz MA, Dey BK, Layer R, Reon BJ, Gagan JR, Dutta A. MUNC, a long noncoding RNA that facilitates the function of MyoD in skeletal myogenesis. Mol Cell Biol. 2015 Feb;35(3):498-513. doi: 10.1128/MCB.01079-14. Epub 2014 Nov 17. PMID: 25403490; PMCID: PMC4285423.

Nagasaka R, Matsumoto M, Okada M, Sasaki H, Kanie K, Kii H, Uozumi T, Kiyota Y, Honda H, Kato R. Visualization of morphological categories of colonies for monitoring of effect on induced pluripotent stem cell culture status. Regen Ther. 2017 Feb 10;6:41-51. doi: 10.1016/j.reth.2016.12.003. PMID: 30271838; PMCID: PMC6134894.

Nakagawa S, Naganuma T, Shioi G, Hirose T. Paraspeckles are subpopulation-specific nuclear bodies that are not essential in mice. J Cell Biol. 2011 Apr 4;193(1):31-9. doi: 10.1083/jcb.201011110. Epub 2011 Mar 28. PMID: 21444682; PMCID: PMC3082198.

Nakayama KI, Clohessy JG, Pandolfi PP. mTORC1 and muscle regeneration are regulated by the LINC00961-encoded SPAR polypeptide. Nature. 2017 Jan 12;541(7636):228-232. doi: 10.1038/nature21034. Epub 2016 Dec 26. PMID: 28024296.

Neppl RL, Wu CL, Walsh K. lncRNA Chronos is an aging-induced inhibitor of muscle hypertrophy. J Cell Biol. 2017 Nov 6;216(11):3497-3507. doi: 10.1083/jcb.201612100. Epub 2017 Aug 30. PMID: 28855249; PMCID: PMC5674882.

Nishimoto Y, Nakagawa S, Hirose T, Okano HJ, Takao M, Shibata S, Suyama S, Kuwako K, Imai T, Murayama S, Suzuki N, Okano H. The long non-coding RNA nuclear-enriched abundant transcript 1_2 induces paraspeckle formation in the motor neuron during the early phase of amyotrophic lateral sclerosis. Mol Brain. 2013 Jul 8;6:31. doi: 10.1186/1756-6606-6-31. PMID: 23835137; PMCID: PMC3729541.

Nishimoto Y, Nakagawa S, Okano H. NEAT1 lncRNA and amyotrophic lateral sclerosis. Neurochem Int. 2021 Nov;150:105175. doi: 10.1016/j.neuint.2021.105175. Epub 2021 Sep 2. PMID: 34481908.

Ogaki S, Morooka M, Otera K, Kume S. A cost-effective system for differentiation of intestinal epithelium from human induced pluripotent stem cells. Sci Rep. 2015 Nov 30;5:17297. doi: 10.1038/srep17297. PMID: 26616277; PMCID: PMC4663490.

Okamoto K, Fujita Y, Mizuno Y. Pathology of protein synthesis and degradation systems in ALS. Neuropathology. 2010 Apr;30(2):189-93. doi: 10.1111/j.1440-1789.2009.01088.x. Epub 2010 Jan 19. PMID: 20102523.

Otomo A, Pan L, Hadano S. Dysregulation of the autophagy-endolysosomal system in amyotrophic lateral sclerosis and related motor neuron diseases. Neurol Res Int. 2012;2012:498428. doi: 10.1155/2012/498428. Epub 2012 Jul 17. PMID: 22852081; PMCID: PMC3407648.

Paulin D, Li Z. Desmin: a major intermediate filament protein essential for the structural integrity and function of muscle. Exp Cell Res. 2004 Nov 15;301(1):1-7.doi:10.1016/j.yexcr.2004.08.004. PMID: 15501438.

Peggion C, Massimino ML, Biancotto G, Angeletti R, Reggiani C, Sorgato MC, Bertoli A, Stella R. Absolute quantification of myosin heavy chain isoforms by selected reaction monitoring can underscore skeletal muscle changes in a mouse model of amyotrophic lateral sclerosis. Anal Bioanal Chem. 2017 Mar;409(8):2143-2153. doi: 10.1007/s00216-016-0160-2. Epub 2017 Jan 11. PMID: 28078418.

Petrou P, Gothelf Y, Argov Z, Gotkine M, Levy YS, Kassis I, Vaknin-Dembinsky A, Ben-Hur T, Offen D, Abramsky O, Melamed E, Karussis D. Safety and Clinical Effects of Mesenchymal Stem Cells Secreting Neurotrophic Factor Transplantation in Patients With Amyotrophic Lateral Sclerosis: Results of Phase 1/2 and 2a Clinical Trials. JAMA Neurol. 2016 Mar;73(3):337-44. doi: 10.1001/jamaneurol.2015.4321. PMID: 26751635.

Picher-Martel V, Valdmanis PN, Gould PV, Julien JP, Dupré N. From animal models to human disease: a genetic approach for personalized medicine in

ALS. Acta Neuropathol Commun. 2016 Jul 11;4(1):70. doi: 10.1186/s40478-016-0340-5. PMID: 27400686; PMCID: PMC4940869.

Postepska-Igielska A, Giwojna A, Gasri-Plotnitsky L, Schmitt N, Dold A, Ginsberg D, Grummt I. LncRNA Khps1 Regulates Expression of the Proto-oncogene SPHK1 via Triplex-Mediated Changes in Chromatin Structure. Mol Cell. 2015 Nov 19;60(4):626-36. doi: 10.1016/j.molcel.2015.10.001. PMID: 26590717.

Pradat PF, Barani A, Wanschitz J, Dubourg O, Lombès A, Bigot A, Mouly V, Bruneteau G, Salachas F, Lenglet T, Meininger V, Butler-Browne G. Abnormalities of satellite cells function in amyotrophic lateral sclerosis. Amyotroph Lateral Scler. 2011 Jul;12(4):264-71. doi: 10.3109/17482968.2011.566618. Epub 2011 Apr 8. PMID: 21473708.

Pradeepa MM, McKenna F, Taylor GC, Bengani H, Grimes GR, Wood AJ, Bhatia S, Bickmore WA. Psip1/p52 regulates posterior Hoxa genes through activation of lncRNA Hottip. PLoS Genet. 2017 Apr 6;13(4):e1006677. doi: 10.1371/journal.pgen.1006677. PMID: 28384324; PMCID: PMC5383017.

Ragagnin AMG, Shadfar S, Vidal M, Jamali MS, Atkin JD. Motor Neuron Susceptibility in ALS/FTD. Front Neurosci. 2019 Jun 27;13:532. doi: 10.3389/fnins.2019.00532. PMID: 31316328; PMCID: PMC6610326.

Raimondi A, Mangolini A, Rizzardini M, Tartari S, Massari S, Bendotti C, Francolini M, Borgese N, Cantoni L, Pietrini G. Cell culture models to investigate the selective vulnerability of motoneuronal mitochondria to familial ALS-linked G93ASOD1. Eur J Neurosci. 2006 Jul;24(2):387-99. doi: 10.1111/j.1460-9568.2006.04922.x. PMID: 16903849.

Ralph GS, Radcliffe PA, Day DM, Carthy JM, Leroux MA, Lee DC, Wong LF, Bilsland LG, Greensmith L, Kingsman SM, Mitrophanous KA, Mazarakis ND, Azzouz M. Silencing mutant SOD1 using RNAi protects against neurodegeneration and extends survival in an ALS model. Nat Med. 2005 Apr;11(4):429-33. doi: 10.1038/nm1205. Epub 2005 Mar 13. PMID: 15768029.

Ransohoff JD, Wei Y, Khavari PA. The functions and unique features of long intergenic non-coding RNA. Nat Rev Mol Cell Biol. 2018 Mar;19(3):143-157. doi: 10.1038/nrm.2017.104. Epub 2017 Nov 15. PMID: 29138516; PMCID: PMC5889127.

Rink J, Ghigo E, Kalaidzidis Y, Zerial M. Rab conversion as a mechanism of progression from early to late endosomes. Cell. 2005 Sep 9;122(5):735-49. doi: 10.1016/j.cell.2005.06.043. PMID: 16143105.

Rocha MC, Pousinha PA, Correia AM, Sebastião AM, Ribeiro JA. Early changes of neuromuscular transmission in the SOD1(G93A) mice model of ALS start long before motor symptoms onset. PLoS One. 2013 Sep 5;8(9):e73846. doi: 10.1371/journal.pone.0073846. PMID: 24040091; PMCID: PMC3764017.

Ross CA, Poirier MA. Protein aggregation and neurodegenerative disease. Nat Med. 2004 Jul;10 Suppl:S10-7. doi: 10.1038/nm1066. PMID: 15272267.

Said Ahmed M, Hung WY, Zu JS, Hockberger P, Siddique T. Increased reactive oxygen species in familial amyotrophic lateral sclerosis with mutations in SOD1. J Neurol Sci. 2000 Jun 15;176(2):88-94. doi: 10.1016/s0022-510x(00)00317-8. PMID: 10930589.

Salameh A, Lee AK, Cardó-Vila M, Nunes DN, Efstathiou E, Staquicini FI, Dobroff AS, Marchiò S, Navone NM, Hosoya H, Lauer RC, Wen S, Salmeron CC, Hoang A, Newsham I, Lima LA, Carraro DM, Oliviero S, Kolonin MG, Sidman RL, Do KA, Troncoso P, Logothetis CJ, Brentani RR, Calin GA, Cavenee WK, Dias-Neto E, Pasqualini R, Arap W. PRUNE2 is a human prostate cancer suppressor regulated by the intronic long noncoding RNA PCA3. Proc Natl Acad Sci U S A. 2015 Jul 7;112(27):8403-8. doi: 10.1073/pnas.1507882112. Epub 2015 Jun 15. PMID: 26080435; PMCID: PMC4500257.

Salmena L, Poliseno L, Tay Y, Kats L, Pandolfi PP. A ceRNA hypothesis: the Rosetta Stone of a hidden RNA language? Cell. 2011 Aug 5;146(3):353-8. doi: 10.1016/j.cell.2011.07.014. Epub 2011 Jul 28. PMID: 21802130; PMCID: PMC3235919.

Sathasivam S, Ince PG, Shaw PJ. Apoptosis in amyotrophic lateral sclerosis: a review of the evidence. Neuropathol Appl Neurobiol. 2001 Aug;27(4):257-74. doi: 10.1046/j.0305-1846.2001.00332.x. PMID: 11532157.

Scaramozza A, Marchese V, Papa V, Salaroli R, Sorarù G, Angelini C, Cenacchi G. Skeletal muscle satellite cells in amyotrophic lateral sclerosis. Ultrastruct Pathol. 2014 Oct;38(5):295-302. doi: 10.3109/01913123.2014.937842. Epub 2014 Jul 31. PMID: 25079897.

Scaricamazza S, Salvatori I, Ferri A, Valle C. Skeletal Muscle in ALS: An Unappreciated Therapeutic Opportunity? Cells. 2021 Mar 2;10(3):525. doi: 10.3390/cells10030525. PMID: 33801336; PMCID: PMC8000428.

Schiaffino S, Reggiani C. Fiber types in mammalian skeletal muscles. Physiol Rev. 2011 Oct;91(4):1447-531. doi: 10.1152/physrev.00031.2010. PMID: 22013216.

Schiaffino S, Dyar KA, Ciciliot S, Blaauw B, Sandri M. Mechanisms regulating skeletal muscle growth and atrophy. FEBS J. 2013 Sep;280(17):4294-314. doi: 10.1111/febs.12253. Epub 2013 Apr 17. PMID: 23517348.

Schiaffino S., Rossi AC, Smerdu V, Leinwand LA, Reggiani C. Developmental myosins: expression patterns and functional significance. Skelet Muscle. 2015 Jul 15;5:22. doi: 10.1186/s13395-015-0046-6. PMID: 26180627; PMCID: PMC4502549.

Shannon P, Markiel A, Ozier O, Baliga NS, Wang JT, Ramage D, Amin N, Schwikowski B, Ideker T. Cytoscape: a software environment for integrated models of biomolecular interaction networks. Genome Res. 2003 Nov;13(11):2498-504. doi: 10.1101/gr.1239303. PMID: 14597658; PMCID: PMC403769.

Shelkovnikova TA, Kukharsky MS, An H, Dimasi P, Alexeeva S, Shabir O, Heath PR, Buchman VL. Protective paraspeckle hyper-assembly downstream of TDP-43 loss of function in amyotrophic lateral sclerosis. Mol Neurodegener. 2018 Jun 1;13(1):30. doi: 10.1186/s13024-018-0263-7. PMID: 29859124; PMCID: PMC5984788.

Smith EF, Shaw PJ, De Vos KJ. The role of mitochondria in amyotrophic lateral sclerosis. Neurosci Lett. 2019 Sep 25;710:132933. doi: 10.1016/j.neulet.2017.06.052. Epub 2017 Jun 30. PMID: 28669745.

Song C, Wang J, Ma Y, Yang Z, Dong D, Li H, Yang J, Huang Y, Plath M, Ma Y, Chen H. Linc-smad7 promotes myoblast differentiation and muscle regeneration via sponging miR-125b. Epigenetics. 2018;13(6):591-604. doi: 10.1080/15592294.2018.1481705. Epub 2018 Aug 6. PMID: 29912619; PMCID: PMC6140903.

Sproviero W, Shatunov A, Stahl D, Shoai M, van Rheenen W, Jones AR, Al-Sarraj S, Andersen PM, Bonini NM, Conforti FL, Van Damme P, Daoud H, Del Mar Amador M, Fogh I, Forzan M, Gaastra B, Gellera C, Gitler AD, Hardy J, Fratta P, La Bella V, Le Ber I, Van Langenhove T, Lattante S, Lee YC, Malaspina A, Meininger V, Millecamps S, Orrell R, Rademakers R, Robberecht W, Rouleau G, Ross OA, Salachas F, Sidle K, Smith BN, Soong BW, Sorarù G, Stevanin G, Kabashi E, Troakes C, van Broeckhoven C, Veldink JH, van den Berg LH, Shaw CE, Powell JF, Al-Chalabi A. ATXN2 trinucleotide repeat length correlates with risk of ALS. Neurobiol Aging. 2017 Mar;51:178.e1-178.e9. doi: 10.1016/j.neurobiolaging.2016.11.010. Epub 2016 Nov 24. PMID: 28017481; PMCID: PMC5302215.

St Laurent G, Wahlestedt C, Kapranov P. The Landscape of long noncoding RNA classification. Trends Genet. 2015 May;31(5):239-51. doi: 10.1016/j.tig.2015.03.007. Epub 2015 Apr 10. PMID: 25869999; PMCID: PMC4417002.

Statello L, Guo CJ, Chen LL, Huarte M. Gene regulation by long non-coding RNAs and its biological functions. Nat Rev Mol Cell Biol. 2021 Feb;22(2):96-118. doi: 10.1038/s41580-020-00315-9. Epub 2020 Dec 22. Erratum in: Nat Rev Mol Cell Biol. 2021 Jan 8;: PMID: 33353982; PMCID: PMC7754182.

Sui Y, Han Y, Zhao X, Li D, Li G. Long non-coding RNA Irm enhances myogenic differentiation by interacting with MEF2D. Cell Death Dis. 2019 Feb 21;10(3):181. doi: 10.1038/s41419-019-1399-2. PMID: 30792383; PMCID: PMC6385193.

Sun L, Si M, Liu X, Choi JM, Wang Y, Thomas SS, Peng H, Hu Z. Long-noncoding RNA Atrolnc-1 promotes muscle wasting in mice with chronic kidney disease. J Cachexia Sarcopenia Muscle. 2018 Oct;9(5):962-974. doi: 10.1002/jcsm.12321. Epub 2018 Jul 24. PMID: 30043444; PMCID: PMC6204593.

Sunwoo H, Dinger ME, Wilusz JE, Amaral PP, Mattick JS, Spector DL. MEN epsilon/beta nuclear-retained non-coding RNAs are up-regulated upon muscle differentiation and are essential components of paraspeckles. Genome Res. 2009 Mar;19(3):347-59. doi: 10.1101/gr.087775.108. Epub 2008 Dec 22. PMID: 19106332; PMCID: PMC2661813.

Swartz EW, Baek J, Pribadi M, Wojta KJ, Almeida S, Karydas A, Gao FB, Miller BL, Coppola G. A Novel Protocol for Directed Differentiation of C9orf72-Associated Human Induced Pluripotent Stem Cells Into Contractile Skeletal Myotubes. Stem Cells Transl Med. 2016 Nov;5(11):1461-1472. doi: 10.5966/sctm.2015-0340. Epub 2016 Jul 1. PMID: 27369896; PMCID: PMC5070503.

Sweeney HL, Hammers DW. Muscle Contraction. Cold Spring Harb Perspect Biol. 2018 Feb 1;10(2):a023200. doi: 10.1101/cshperspect.a023200. PMID: 29419405; PMCID: PMC5793755.

Sweta S, Dudnakova T, Sudheer S, Baker AH, Bhushan R. Importance of Long Non-coding RNAs in the Development and Disease of Skeletal Muscle and Cardiovascular Lineages. Front Cell Dev Biol. 2019 Oct 18;7:228. doi: 10.3389/fcell.2019.00228. PMID: 31681761; PMCID: PMC6813187.

Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, Yamanaka S. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell. 2007 Nov 30;131(5):861-72. doi: 10.1016/j.cell.2007.11.019. PMID: 18035408.

Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell. 2006 Aug 25;126(4):663-76. doi: 10.1016/j.cell.2006.07.024. Epub 2006 Aug 10. PMID: 16904174.

Telerman-Toppet N, Coërs C. Motor innervation and fiber type pattern in amyotrophic lateral sclerosis and in Charcot-Marie-Tooth disease. Muscle Nerve. 1978 Mar-Apr;1(2):133-9. doi: 10.1002/mus.880010205. PMID: 155778.

Tripathi V, Ellis JD, Shen Z, Song DY, Pan Q, Watt AT, Freier SM, Bennett CF, Sharma A, Bubulya PA, Blencowe BJ, Prasanth SG, Prasanth KV. The nuclear-retained noncoding RNA MALAT1 regulates alternative splicing by modulating SR splicing factor phosphorylation. Mol Cell. 2010 Sep 24;39(6):925-38. doi: 10.1016/j.molcel.2010.08.011. PMID: 20797886; PMCID: PMC4158944.

Tsai MC, Manor O, Wan Y, Mosammaparast N, Wang JK, Lan F, Shi Y, Segal E, Chang HY. Long noncoding RNA as modular scaffold of histone modification complexes. Science. 2010 Aug 6;329(5992):689-93. doi: 10.1126/science.1192002. Epub 2010 Jul 8. PMID: 20616235; PMCID: PMC2967777.

Tsitkanou S, Lindsay A, Della Gatta P. The role of skeletal muscle in amyotrophic lateral sclerosis: a 'dying-back' or 'dying-forward' phenomenon? J Physiol. 2019 Dec;597(23):5527-5528. doi: 10.1113/JP278835. Epub 2019 Nov 2. PMID: 31605390.

Wang GQ, Wang Y, Xiong Y, Chen XC, Ma ML, Cai R, Gao Y, Sun YM, Yang GS, Pang WJ. Sirt1 AS lncRNA interacts with its mRNA to inhibit muscle formation by attenuating function of miR-34a. Sci Rep. 2016 Feb 23;6:21865. doi: 10.1038/srep21865. PMID: 26902620; PMCID: PMC4763196.

Wang J, Liu X, Wu H, Ni P, Gu Z, Qiao Y, Chen N, Sun F, Fan Q. CREB up-regulates long non-coding RNA, HULC expression through interaction with microRNA-372 in liver cancer. Nucleic Acids Res. 2010 Sep;38(16):5366-83. doi: 10.1093/nar/gkq285. Epub 2010 Apr 27. PMID: 20423907; PMCID: PMC2938198.

Wang KC, Yang YW, Liu B, Sanyal A, Corces-Zimmerman R, Chen Y, Lajoie BR, Protacio A, Flynn RA, Gupta RA, Wysocka J, Lei M, Dekker J, Helms JA, Chang HY. A long noncoding RNA maintains active chromatin to coordinate homeotic gene expression. Nature. 2011 Apr 7;472(7341):120-4. doi: 10.1038/nature09819. Epub 2011 Mar 20. PMID: 21423168; PMCID: PMC3670758.

Wang S, Jin J, Xu Z, Zuo B. Functions and Regulatory Mechanisms of lncRNAs in Skeletal Myogenesis, Muscle Disease and Meat Production. Cells. 2019 Sep 19;8(9):1107. doi: 10.3390/cells8091107. PMID: 31546877; PMCID: PMC6769631.

Wang S, Zuo H, Jin J, Lv W, Xu Z, Fan Y, Zhang J, Zuo B. Long noncoding RNA NEAT1 modulates myogenesis by recruiting Ezh2. Cell Death Dis. 2019 Jun 26;10(7):505. doi: 10.1038/s41419-019-1742-7. PMID: 31243262; PMCID: PMC6594961.

Watts R, Johnsen VL, Shearer J, Hittel DS. Myostatin-induced inhibition of the long noncoding RNA MALAT1 is associated with decreased myogenesis. Am J Physiol Cell Physiol. 2013 May 15;304(10):C995-1001. doi: 10.1152/ajpcell.00392.2012. Epub 2013 Mar 13. PMID: 23485710.

Xiao Y, Ma C, Yi J, Wu S, Luo G, Xu X, Lin PH, Sun J, Zhou J. Suppressed autophagy flux in skeletal muscle of an amyotrophic lateral sclerosis mouse model during disease progression. Physiol Rep. 2015 Jan 19;3(1):e12271. doi: 10.14814/phy2.12271. PMID: 25602021; PMCID: PMC4387765.

Yamamoto M, Kuroiwa A. Hoxa-11 and Hoxa-13 are involved in repression of MyoD during limb muscle development. Dev Growth Differ. 2003 Oct-

Dec;45(5-6):485-98. doi: 10.1111/j.1440-169x.2003.00715.x. PMID: 14706073.

Yang L, Lin C, Liu W, Zhang J, Ohgi KA, Grinstein JD, Dorrestein PC, Rosenfeld MG. ncRNA- and Pc2 methylation-dependent gene relocation between nuclear structures mediates gene activation programs. Cell. 2011 Nov 11;147(4):773-88. doi: 10.1016/j.cell.2011.08.054. Erratum in: Cell. 2013 Oct 10;155(2):478. PMID: 22078878; PMCID: PMC3297197.

Yeh TH, Liu HF, Li YW, Lu CS, Shih HY, Chiu CC, Lin SJ, Huang YC, Cheng YC. C9orf72 is essential for neurodevelopment and motility mediated by Cyclin G1. Exp Neurol. 2018 Jun;304:114-124. doi: 10.1016/j.expneurol.2018.03.002. Epub 2018 Mar 6. PMID: 29522758.

Yu X, Zhang Y, Li T, Ma Z, Jia H, Chen Q, Zhao Y, Zhai L, Zhong R, Li C, Zou X, Meng J, Chen AK, Puri PL, Chen M, Zhu D. Long non-coding RNA Linc-RAM enhances myogenic differentiation by interacting with MyoD. Nat Commun. 2017 Jan 16;8:14016. doi: 10.1038/ncomms14016. PMID: 28091529; PMCID: PMC5241866.

Zammit PS, Partridge TA, Yablonka-Reuveni Z. The skeletal muscle satellite cell: the stem cell that came in from the cold. J Histochem Cytochem. 2006 Nov;54(11):1177-91. doi: 10.1369/jhc.6R6995.2006. Epub 2006 Aug 9. PMID: 16899758.

Zhang P, Du J, Guo X, Wu S, He J, Li X, Shen L, Chen L, Li B, Zhang J, Xie Y, Niu L, Jiang D, Li X, Zhang S, Zhu L. LncMyoD Promotes Skeletal Myogenesis and Regulates Skeletal Muscle Fiber-Type Composition by Sponging miR-370-3p. Genes (Basel). 2021 Apr 17;12(4):589. doi: 10.3390/genes12040589. PMID: 33920575; PMCID: PMC8072939.

Zhang X, Hamblin MH, Yin KJ. The long noncoding RNA MALAT1: Its physiological and pathophysiological functions. RNA Biol. 2017 Dec 2;14(12):1705-1714. doi: 10.1080/15476286.2017.1358347. Epub 2017 Oct 6. PMID: 28837398; PMCID: PMC5731810.

Zhang ZK, Li J, Guan D, Liang C, Zhuo Z, Liu J, Lu A, Zhang G, Zhang BT. Long Noncoding RNA lncMUMA Reverses Established Skeletal Muscle Atrophy following Mechanical Unloading. Mol Ther. 2018 Nov 7;26(11):2669-2680. doi: 10.1016/j.ymthe.2018.09.014. Epub 2018 Oct 25. PMID: 30415659; PMCID: PMC6225098.

Zhao C, Rajashankar KR, Marcia M, Pyle AM. Crystal structure of group II intron domain 1 reveals a template for RNA assembly. Nat Chem Biol. 2015 Dec;11(12):967-72. doi: 10.1038/nchembio.1949. Epub 2015 Oct 26. PMID: 26502156; PMCID: PMC4651773.

Zheng L, Liu X, Chen P, Xiao W. Expression and role of lncRNAs in the regeneration of skeletal muscle following contusion injury. Exp Ther Med. 2019 Oct;18(4):2617-2627. doi: 10.3892/etm.2019.7871. Epub 2019 Aug 8.

Erratum in: Exp Ther Med. 2020 Jan;19(1):797. PMID: 31572510; PMCID: PMC6755471.

Zhou L, Sun K, Zhao Y, Zhang S, Wang X, Li Y, Lu L, Chen X, Chen F, Bao X, Zhu X, Wang L, Tang LY, Esteban MA, Wang CC, Jauch R, Sun H, Wang H. Linc-YY1 promotes myogenic differentiation and muscle regeneration through an interaction with the transcription factor YY1. Nat Commun. 2015 Dec 11;6:10026. doi: 10.1038/ncomms10026. PMID: 26658965.

Zhou L, Ren M, Zeng T, Wang W, Wang X, Hu M, Su S, Sun K, Wang C, Liu J, Yang C, Yan L. TET2-interacting long noncoding RNA promotes active DNA demethylation of the MMP-9 promoter in diabetic wound healing. Cell Death Dis. 2019 Oct 25;10(11):813. doi: 10.1038/s41419-019-2047-6. PMID: 31653825; PMCID: PMC6814823.

Zhu M, Liu J, Xiao J, Yang L, Cai M, Shen H, Chen X, Ma Y, Hu S, Wang Z, Hong A, Li Y, Sun Y, Wang X. Lnc-mg is a long non-coding RNA that promotes myogenesis. Nat Commun. 2017 Mar 10;8:14718. doi: 10.1038/ncomms14718. PMID: 28281528; PMCID: PMC5353601.

Zhu Z, Pan Q, Zhao W, Wu X, Yu S, Shen Q, Zhang J, Yue W, Peng S, Li N, Zhang S, Lei A, Hua J. BCL2 enhances survival of porcine pluripotent stem cells through promoting FGFR2. Cell Prolif. 2021 Jan;54(1):e12932. doi: 10.1111/cpr.12932. Epub 2020 Oct 26. PMID: 33107129; PMCID: PMC7791183.

Acknowledgements

I would like to thank Doctor Renato Emilio Mantegazza for giving me the opportunity to work in his Operative Unit and to develop multiple skills in the research field. Thanks also to Pia, who will always be a pillar in my research career.

Thanks to Professor Francesco Saverio Tadesco for the ospitality in his laboratory at The Francis Crick Institute in London and for teaching me how to work in a multi-ethnic environment.

An infinite thank to Stefi and Silvi for shaping me as a researcher and, more than all the rest, as a person. Without your competences, technical and moral support, intuition and never ending enthusiasm, this work would not exhist.

Thank you Claudia for sharing with me this PhD journey. Your empathy and truthfulness made my work not a work. I could not ask for a better partner in crime. E che il disagio ci accompagni sempre.

Thanks to the old and new members of the lab, Chiara, Cristina, Federica, Fulvia, Letizia, Marco, Maria Cristina, Marta, Nicola and Paola for the help, support and fun in the lab.

Thanks to the new friends, more than collegues, that I found in London, Cathy, Daniele, Elisa, Giulia, Luca and Salma. A special kiss for Luca (volpino), Moufi (Moustafa, Moufi, Moufino, Moustafino, Moufasa), Noreena (eeeeeeeeeeh???), Sunghino (even if you're dead inside I still love you) and Vale (you're just unique) for being the best Londoners.

To my mum, dad and brother. For giving me love, encouragement, strength and tenacy. Thanks for all the values you gave me and for all the things you still have the patient and kindness to teach me everyday. Your love and support are and always be fundmental.

Infinite thanks to Bice, the love of my life. Thanks for being my sister and best friend. You taught me how to love inconditionally.

… and even if you still are after Bice, thanks to all the rest of my family! You all contributed to grow me up and to shape me with all the amazing values you have.

Thank you Ste. Even if you walked with me for a limited period, you will always be in my thoughts and heart. Without you, my life would have gone in a completely different direction.

Thanks Richi (Babe!!) because you gave me relief, happiness, fun, struggle and, more than anything, the courage for seeking the change I never found by myself. I hope we will share more adventures together, that you will still have the patient to listen to me, to make me discover the world and myself everyday, and to help me in changing again and again and again.