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Investigating the molecular impact of *S/L1* mutations in Marinesco-Sjögren Syndrome

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ABSTRACT

Marinesco-Sjögren syndrome (MSS) is a rare, multisystemic autosomal recessive disorder that arises in early childhood. Main symptoms include cerebellar ataxia, myopathy and cataracts, but patients can also manifest mental retardation, short stature, skeletal deformities and nystagmus. MSS is primarily caused by mutations in the *SIL1* gene, which encodes for an endoplasmic reticulum (ER) co-chaperone essential for BiP-mediated protein folding. Defective Sil1 protein disrupts protein homeostasis within the ER, leading to the accumulation of misfolded proteins and the subsequent activation of the unfolded protein response (UPR). Prolonged UPR most severely affects Purkinje cells and skeletal muscle fibers, but other cell types are also susceptible to its detrimental effects. However, the precise molecular pathways involved in the pathogenesis of MSS remain to be elucidated.

This thesis integrates *in vitro* and *in vivo* investigations to clarify the molecular consequences of Sil1 loss. Cellular and proteomic analysis of fibroblasts derived from an MSS patient carrying the R111X mutation revealed a mild UPR activation, accompanied by pronounced metabolic reprogramming. These alterations include downregulation of RNA processing and translation machineries, upregulation of lysosomal protein degradation, and shifts in lipid and amino acid metabolism, highlighting adaptive cellular mechanisms to Sil1 loss.

Furthermore, transcriptomic and morphological analyses demonstrated alterations in extracellular matrix (ECM) remodelling and cell adhesion processes, suggesting that a dysfunctional ECM could play a central role in the development of the broad clinical spectrum of MSS.

Additionally, studies on the novel *SIL1* variant (p.E342K) provided insights into genotype-phenotype relationships and confirmed pathological changes consistent with MSS. The novel *SIL1* variant (p.E342K) identified in a patient exhibiting the hallmark triad of MSS symptoms, was functionally validated as pathogenic through *in silico* predictions and structural analysis of the recombinant protein. Patient-derived fibroblasts also displayed the presence of an established MSS cellular phenotype, and reduced protein levels of mutated Sil1, which could be rescued by proteasome inhibition.

Finally, the *woozy* (*Sil1^{wz}*) mouse model offered crucial insights into MSS progression, revealing sex-specific differences, vulnerability of glycolytic muscles, and cognitive deficits akin to mental disability seen in MSS patients. This detailed characterisation highlighted the value of preclinical models for MSS and for the development of targeted therapies.

Collectively, these findings improved our understanding of MSS and the systemic consequences of *SIL1* deficiency.

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1. Introduction

1.1 *Sil1* protein

The human *SIL1* gene is located on chromosome 5q31.2 and it encodes a 54 kDa glycoprotein that resides in the endoplasmic reticulum (ER). Sil1 protein acts as a co-chaperone and nucleotide exchange factor (NEF) for the HSP70 chaperone BiP (Binding immunoglobulin protein, also referred to as 78-kDa glucose-regulated protein GRP78), which plays crucial roles in the ER [1]. In particular, BiP interacts with newly synthesised proteins to ensure their correct folding, but it also promotes the degradation of aberrant proteins by translocating them across the ER membranes, towards the proteasome. Moreover, BiP acts as a stress sensor within the ER, and it is upregulated when many misfolded proteins accumulate [2]. The functional activity of BiP depends on its ATPase cycle, in which Sil1 participates in promoting the ADP-ATP exchange, thus facilitating the release of the folded substrate (Figure 1) [3]. *SIL1* was identified for the first time in two different species of yeast. In *Yarrowia lipolytica*, the homologous of this protein (Sls1p) resides in the lumen of the ER, is involved in protein transport, and is able to interact with a chaperone deputed to protein folding (Kar2p, the equivalent of BiP) [4, 5]. In *Saccharomyces cerevisiae*, Sil1p (suppressor of Ire1 and Lhs1 deletion 1) exhibits homology to Sls1p and is vital for the translocation of proteins across the ER membrane [6]. In mammals, Sil1 is present in all tissues, but different expressions of this protein are strictly correlated to BiP amount. This correlation is not as evident for the ER chaperone GRP170 (also known as ORP150), which is another NEF for BiP [7, 8]. Several mutations in the *SIL1* gene, resulting in Sil1 loss, have been associated with the majority of patients affected by Marinesco-Sjögren Syndrome (MSS).

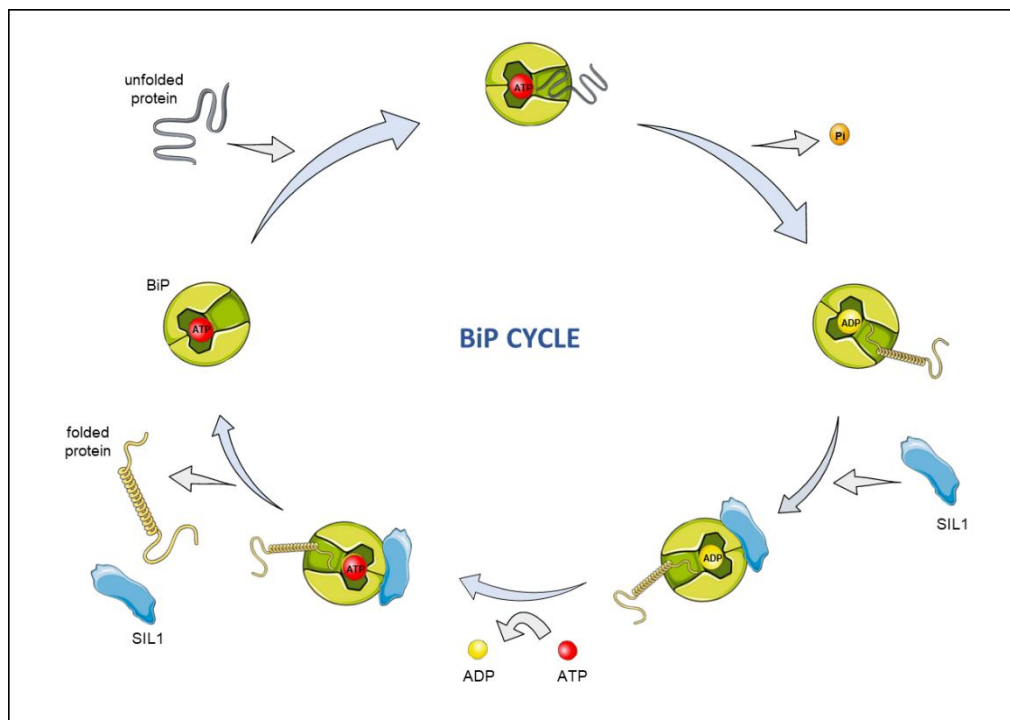


Figure 1 The ATPase cycle of the endoplasmic reticulum (ER) chaperone BiP facilitates client protein folding. Binding of ATP to BiP enables the recognition and association with nascent unfolded polypeptide chains entering the ER lumen. Subsequent ATP hydrolysis induces a conformational change in BiP, stabilising its interaction with the client protein and promoting its folding. The nucleotide exchange factor Sil1 catalyses the replacement of ADP with ATP, leading to the release of the correctly folded protein from BiP. In the absence of functional Sil1, the ADP-bound BiP remains stably associated with its substrate, impeding the chaperone cycle and resulting in the accumulation of unfolded proteins within the ER.

From “Genotype-phenotype correlation and adaptive proteome reorganization in Marinesco-Sjögren syndrome”. Ruggieri A.G. and Salles M., 2022. DOI: 10.36253/ijae-13802.

1.2 Marinesco-Sjögren Syndrome

Initially described by Gheorghe Marinesco in the 1930s and later elaborated by Torsten Sjögren in 1950, Marinesco-Sjögren Syndrome (MSS; OMIM 248800) is defined as a distinct clinical condition with unique pathophysiological characteristics [9, 10]. This extremely rare neurogenetic disease manifests during early childhood and affects approximately one in a million individuals. About 60% of cases result from autosomal recessive inheritance of the mutated *SIL1* gene. The three hallmark symptoms, essential for diagnosis, include cerebellar ataxia, myopathy, and bilateral cataracts [11].

Cataracts, frequently bilateral and congenital or appearing shortly after birth, are usually the initial symptoms. Their rapid progression often necessitates lens removal surgery by approximately age ten. In addition, patients could exhibit nystagmus and strabismus (Figure 2) [12].

Hypotonia and muscle weakness give rise to the so-called "floppy baby" condition in affected children, who also experience myopathy and delayed psychomotor development. Muscle fibers degeneration is evident, and biopsy analysis typically reveals altered fiber size and composition, in addition to replacement with fibroadipose tissue [13, 14]. Motor functions are compromised during growth until a plateau is reached, with timing and severity differing across cases. However, most patients eventually require walking aids [15].

Neurological damage is highlighted by Purkinje neurons degeneration, which leads to ataxia, balance disturbances, and cerebellar atrophy. Other manifestations related to cerebellar lesions are incoordination of muscle groups (dyssynergia), inaccurate movements (dysmetria), difficulty in rapid alternating movements (dysdiadochokinesia), and impaired stance and gait [16].

Patients may also exhibit learning disabilities, intellectual impairment, language difficulties, intention tremors, hypergonadotropic hypogonadism, short stature, and skeletal deformities.

Despite being a debilitating multi-organ disease, MSS does not seem to shorten lifespan.

Its diagnosis relies on a clinical evaluation of key symptoms—using magnetic resonance imaging to detect cerebellar atrophy, ophthalmologic examinations to identify cataracts, and muscle biopsies to confirm myopathy—with genetic tests for biallelic *SIL1* mutations supporting these findings. [15].

Currently, there is no cure for MSS; surgery can correct cataracts, but treatment for other symptoms is limited to supportive therapies. Hormone therapy addresses hypergonadotropic hypogonadism and helps prevent osteoporosis, while neurologists and physical therapists typically manage psychomotor difficulties [17].

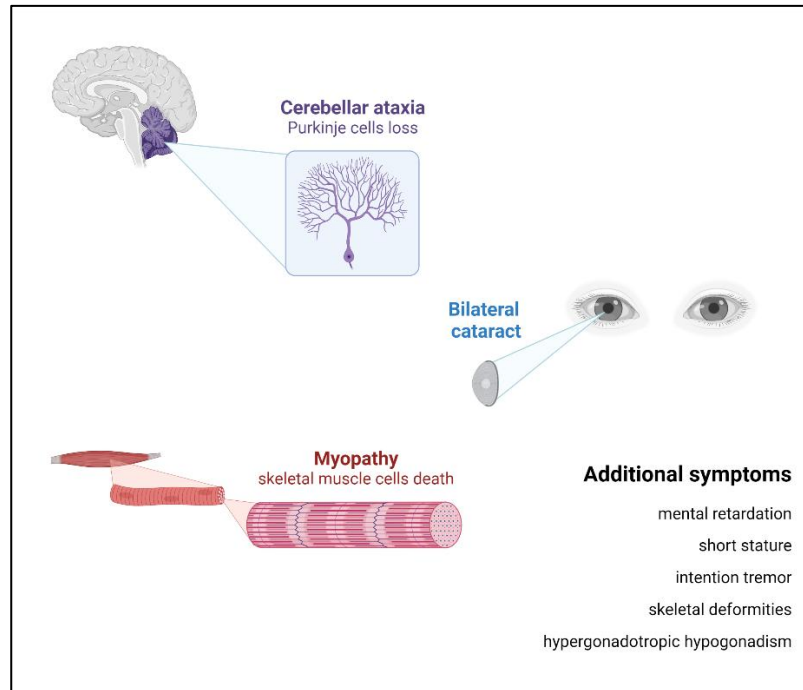


Figure 2 Key clinical features of Marinesco-Sjögren Syndrome.

From “Transcriptomic analysis revealed aberrant extracellular matrix in *SIL1* deficient human fibroblasts”
Poster Ruggieri A.G. et al., 2024 (48th FEBS Congress).

1.3 Molecular Insights into Marinesco-Sjögren Syndrome

The main molecular effect of mutations in the *SIL1* gene in MSS is a disruption of the protein folding machinery. Sil1 mutants are frequently degraded or unable to interact with BiP, thus misfolded proteins accumulate in the ER leading to the activation of the unfolded protein response (UPR) [18].

This complex cellular mechanism works to restore normal ER homeostasis by boosting the production of molecular chaperones to prevent protein aggregation, enhancing proteasomal degradation of abnormal proteins, and reducing protein synthesis. To carry out these functions, three ER transmembrane proteins are activated: inositol-requiring enzyme 1 (IRE1), PRK-like kinase (PERK), and activating transcription factor 6 (ATF6) (Figure 3) [19].

IRE1 has a luminal domain for binding BiP, when it is inactivated, and a cytosolic domain with kinase and endoribonuclease activity. When misfolded proteins accumulate, IRE1 oligomerizes and autophosphorylates, activating its ribonuclease function. This leads to the alternative splicing of XBP1 mRNA, producing the active form sXBP1, which moves to the nucleus to promote the expression of genes involved in ER-associated degradation (ERAD), chaperones, and lipid synthesis. Additionally, IRE1 activates the regulated IRE1-dependent decay (RIDD) pathway to degrade RNA, reducing overall protein synthesis and ER’s protein folding workload

[19, 20].

Concurrently, in response to ER stress, the membrane protein PERK oligomerizes and autophosphorylates, thus leading to the phosphorylation of eIF2 α and the subsequent reduction in overall protein synthesis. However, specific mRNAs, like activating transcription factor 4 (ATF4), are preferentially translated under these conditions. ATF4 translocates into the nucleus and activates UPR gene transcription, including the C/EBP homologous protein (CHOP) and the growth arrest and DNA-damage inducible protein-34 (GADD34). CHOP promotes apoptosis, while GADD34 dephosphorylates eIF2 α , restoring protein synthesis once the ER stress is resolved [19, 20].

ATF6, the third effector of UPR, is a transcription factor initially synthesised as a transmembrane protein in the ER. When misfolded proteins accumulate, ATF6 is transported to the Golgi apparatus, where it is cleaved by two proteases: S1P and S2P. This cleavage releases the cytosolic domain of about 50 kDa, which then translocates into the nucleus to activate UPR genes, including those involved in protein folding, such as BiP and XBP.

The combined molecular responses triggered by IRE1, PERK and ATF6 work to promote cell survival; however, when UPR is prolonged over time, it leads to apoptosis [20].

In MSS, the extent of UPR activation seems to correlate with cellular damage. Specifically, skeletal muscle cells and Purkinje neurons exhibit a strongly activated UPR, whereas cells that are not the primary target of the disease, such as fibroblasts, show only a mild UPR and rely on alternative mechanisms to manage protein overload [17, 21].

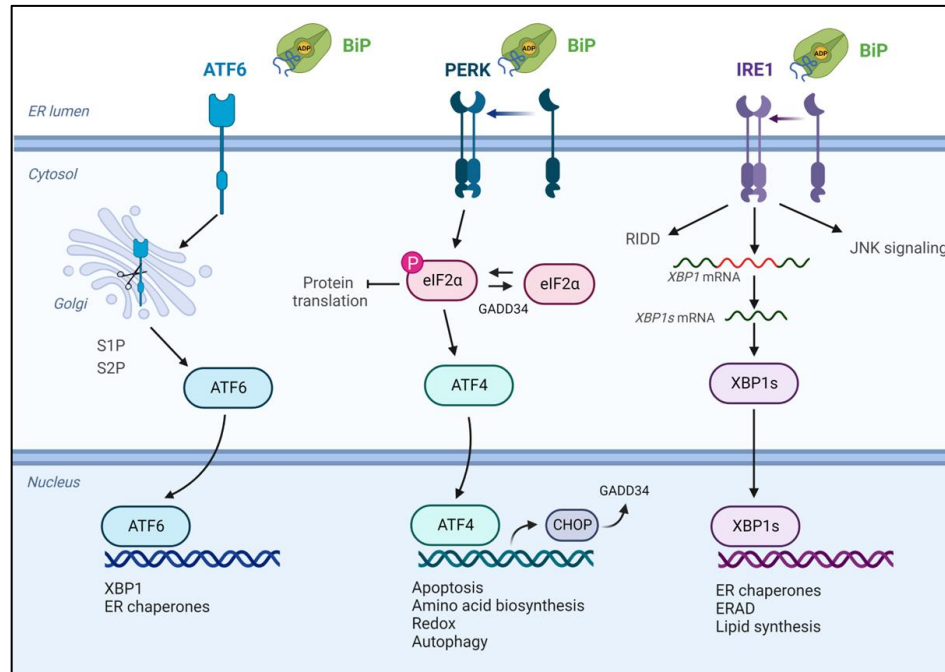


Figure 3 The unfolded protein response (UPR) is a critical cellular stress pathway activated to restore endoplasmic reticulum (ER) homeostasis upon accumulation of misfolded proteins. This defence mechanism is initiated by three ER transmembrane stress sensors: inositol-requiring enzyme 1 (IRE1), activating transcription factor 6 (ATF6), and protein kinase R (PKR)-like endoplasmic reticulum kinase (PERK). Activation of these sensors triggers downstream signalling cascades that collectively aim to enhance the ER's protein folding capacity, promote the degradation of misfolded proteins, and attenuate global protein synthesis to alleviate ER stress.

From "Study of the pathological mechanisms of the Marinesco-Sjögren syndrome by transcriptomic analysis" Poster Ruggieri A.G. et al., 2022 (EMBO Workshop "Endoplasmic Reticulum: The Master Regulator of Membrane Trafficking").

1.4 Sil1-Deficient Animal Models of Marinesco-Sjögren Syndrome

Within the past few years, Sil1-deficient animals have played a crucial role in clarifying the function of Sil1 protein and its involvement in MSS.

Concerning the two existing mouse models, they both exhibit the MSS phenotype due to a specific defect in intron 7 of the murine *Sil1* gene, despite the differences in the resulting chimeric proteins and in the genetic background (CxB5/ByJ vs. C57BL/6J) [22].

Introduced in 2005, the first model—known as the *woozy* (*wz*) mouse (*Sil1^{wz}*)—spontaneously expresses a truncated Sil1 protein. This occurs due to the insertion of an early transposon element adjacent to exon seven, which results in an additional 96 coding nucleotides followed by a stop codon immediately after the retrotransposon coding sequence [23]. Mutated Sil1 protein leads to significant protein accumulation and disruption of ER homeostasis, which triggers neurodegenerative processes and ataxia. From 6 weeks of age *woozy* mice exhibit signs

of UPR activation in Purkinje cells, as indicated by the upregulation of BiP, CHOP and XBP1, and the increased phosphorylation of eIF2 within cerebellar lobules I-VIII, sparing lobule X and part of IX.

Within the 4th month, Purkinje cells undergo apoptosis, as shown by immunofluorescence detection of activated caspase-3 and TUNEL-positive DNA breaks. Furthermore, the presence of condensed nuclear chromatin, autophagosome-like vacuoles and electron-dense structures reminiscent of protein inclusions revealed through electron microscopy, provides additional evidence of the degenerative process of Purkinje cells [23].

Other molecular abnormalities have also been detected in skeletal muscle cells of *woozy* mice, consistent with human MSS myopathy. These alterations affect several subcellular compartments, concurrent with activation of UPR and ERAD pathways. Notably, quadriceps biopsy revealed variability of muscle fiber size, irregular sarcoplasmic reticulum, swollen mitochondria, as well as accumulation of autophagic vacuoles and abnormal perinuclear membranous structures [14].

Visibly, *wz* mice exhibit motor deficits from 10 weeks of age, as demonstrated by rotarod testing from 6 to 34 weeks [24].

According to its resemblance to human MSS, this model is valuable for studying disease mechanisms and developing treatments, as well as the *Sil1^{Gt}* mouse.

This second model was generated from a gene-trap ES cell line with a β -geo insertion in the *Sil1* gene, leading to transcript truncation after exon 7 and thus demonstrating *Sil1* involvement in neurodegeneration and progressive myopathy [23]. A proteomic analysis conducted on the quadriceps of *Sil1^{Gt}* mouse in 2018 by Ichhaporia and colleagues highlighted more than 500 proteins differentially expressed, impacting structure and function of skeletal muscle compared to wild-type (WT) [25]. Additionally, morphological analysis demonstrated irregular muscle fibers and myofibrillar disorganisation with adipose tissue replacement. Despite cellular attempts to counteract muscle atrophy by enhancing PI3K-AKT-mTOR signalling and glucose uptake, these compensatory mechanisms were unable to prevent ER homeostasis disruption. Evidence of reduced muscle strength in *Sil1^{Gt}* mice was apparent from 24 weeks, with a progressively diminishing ability to maintain a grip on an inverted screen as they aged [25]. Notably, in contrast to bilateral cataracts that characterise human MSS symptomatology, *Sil1^{Gt}* mice show only occasional unilateral cataract development [22].

Zebrafish model was also used by researchers to study MSS pathophysiology [26, 27]. To silence *Sil1*, antisense morpholino oligonucleotides were injected into embryos of zebrafish

Danio rerio, thus leading to Purkinje cells loss, as demonstrated by immunohistochemistry, and disrupted muscle fiber organisation, as evidenced by a birefringence assay. Western blot analysis also revealed the presence of ER stress, autophagy and apoptosis, through the upregulation of BiP, lipidated LC3 and activated caspase-3 [26].

Moreover, neuromuscular junctions are compromised by *Sil1* loss, resulting in a disrupted organisation in the zebrafish model, and a significant reduction in size and acetylcholine receptor (AChR) in *woozy* mice, compared to WT controls [27].

Zebrafish also displayed smaller eyes instead of cataracts, thus suggesting a possible role of *Sil1* in eye development [26].

1.5 Therapeutic Approaches

To date, MSS remains without a cure. Like other neurodegenerative diseases, such as Alzheimer's, Parkinson's, amyotrophic lateral sclerosis (ALS) and prion disease, MSS is a misfolding disorder featuring activation of the UPR [28, 29, 30, 31].

Among the UPR cascade pathways, the inhibition of protein synthesis mediated by $\text{p}e\text{IF}2\alpha$ (triggered by PERK activation) is probably the most crucial factor driving synaptic protein depletion and neurodegeneration. To challenge this effect and restore protein synthesis, researchers tried to inhibit PERK activity in an *in vitro* model of MSS and in the *woozy* mice [24, 32]. Cell treatment with PERK inhibitor GSK2606414 improved subcellular structures deputed to secretion and reduced apoptosis, while treated mice exhibited an improvement of motor functions and a delay of neurodegeneration. Purkinje neurons of treated *wz* mice displayed higher levels of ORP150, suggesting a possible compensatory mechanism to assist BiP. Unfortunately, the loss of the beneficial effects of treatment over time and pancreatic toxicity are limitations that merit investigation in order to improve this approach.

A different therapeutic strategy aimed at counteracting the accumulation of misfolded proteins in MSS is represented by using chemical chaperones. These small molecules favour the correct conformation of proteins thanks to electrostatic or hydrophobic bonds [33]. Interestingly, for some human genetic diseases, pharmacological chemical chaperones have already been tested or approved, such as migalastat for Fabry disease, tafamidis for transthyretin amyloidosis, and combinations such as ivacaftor, tezacaftor, and elexacaftor for cystic fibrosis [34, 35, 36]. In the context of MSS, experiments using patient-derived lymphoblastoid cells with the common p.Leu313fs *SIL1* mutation demonstrated that while these cells exhibit ER stress and heightened

cell death upon tunicamycin treatment, the chemical chaperone TUDCA (tauroursodeoxycholic acid) was able to significantly reduce cell death, even though it did not lower stress markers [37]. Thus, the development of Sil1-specific chaperones, possibly combined with proteostasis regulators, could represent a promising future treatment for MSS, but mutation-dependent efficacy is a concern.

Finally, the high frequency of *SIL1* gene mutations in MSS makes gene therapy a particularly attractive treatment strategy. In particular, the reintroduction of a functional *SIL1* gene could correct the underlying cellular dysfunction and potentially alleviate MSS symptoms, as already demonstrated in other similar genetic diseases. For example, in Friedreich ataxia (FRDA) models, viral delivery of human frataxin via HSV or AAV vectors improved motor coordination and cardiac function, while AAV-mediated SMN1 gene reintroduction has dramatically enhanced spinal muscular atrophy outcomes [38, 39]. In conclusion, treating MSS poses challenges due to its systemic nature and its delayed diagnosis represents a limit for treatment efficacy. However, recent investigations into various therapeutic approaches provide a foundation for future improvements (Figure 4).

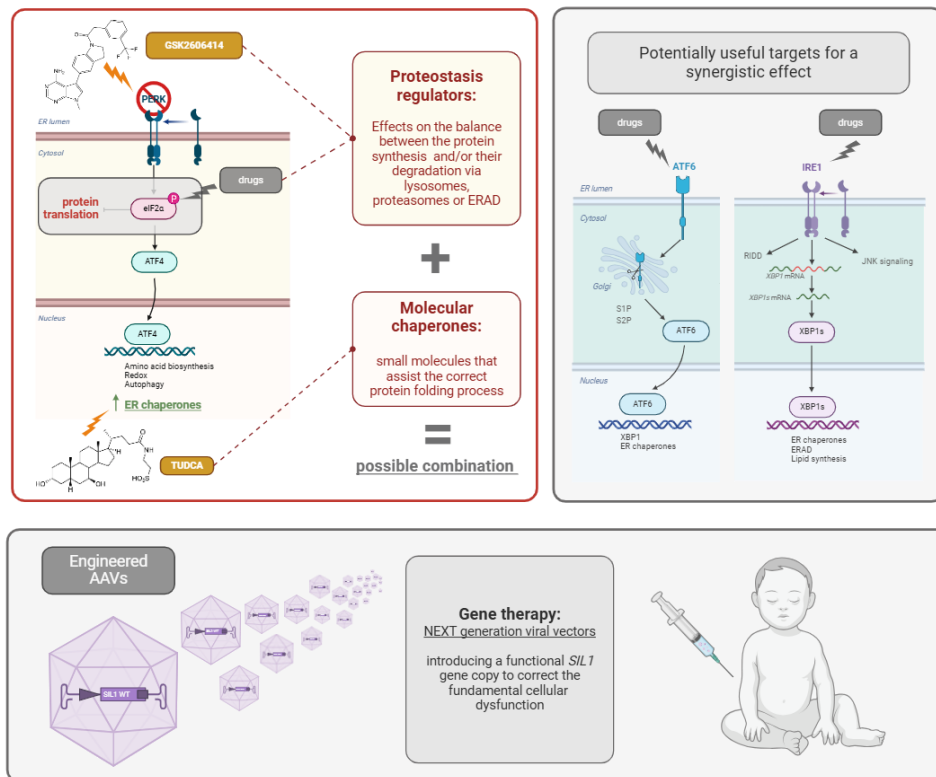


Figure 4 Therapeutic approaches for Marinesco-Sjögren syndrome.
From “Potential approaches for the treatment of Marinesco –Sjögren syndrome”.
Viele M. et al., 2024. DOI: 10.61012_20248_590.

2. Research proposal

The primary aim of my doctoral research was to explore the molecular consequences of *SIL1* mutations in MSS *in vitro* and *in vivo*.

For this purpose, I initially delved into the characterisation of an *in vitro* model of MSS, specifically skin fibroblasts obtained from a patient carrying the R111X mutation (HF-MSS). In particular, we conducted both proteomic and transcriptomic analyses on these cells to gain deeper insights into the pathogenesis of this syndrome. This multi-omics approach combined information about the proteome adaptation to Sil1 loss and the expression of RNA transcripts in response to altered molecular processes, thereby shedding light on key biological processes of MSS and the discovery of new biomarkers.

Secondly, considering the current knowledge and our own findings on the molecular mechanisms of MSS, I have focused my research on a new variant of unknown significance (VUS) of the *SIL1* gene. In collaboration with a Greek research group, we examined a 3-year-old patient presenting with the clinical triad characteristic of MSS and carrying the novel c.1024G>A mutation that results in the p.E342K substitution. Our goal was to determine the pathogenic nature of this VUS, as revealed by multiple in-silico tools, through structural examinations of the mutated protein and cellular and molecular analysis of patient-derived fibroblasts. Furthermore, we compared the proteomic profile of these cells with that of our previously analysed MSS patient and the *Sil1^{Gt}* mouse model.

Ultimately, my research efforts were dedicated to providing a comprehensive phenotypic and molecular characterisation of our *woozy* mice colony, to advance MSS knowledge and future therapy development. We conducted a series of motor tests (rotarod, beam walking, grip strength test) with the aim of accurately defining the progression of ataxia. To explore potential cognitive impairments, we also performed nest building, novel object recognition and Y-maze tests. Finally, we carried out a preliminary biochemical analysis of affected tissues in order to investigate the differential vulnerability of glycolytic versus oxidative muscles.

In conclusion, as a result of these combined *in vitro* and *in vivo* investigations, we have broadened our understanding of the molecular and phenotypic effects of *SIL1* mutations in MSS, paving the way for the identification of potential biomarkers and the development of targeted therapies.

3. Results

3.1 Proteomic Analysis of Marinesco-Sjögren Syndrome Fibroblasts

From “Proteomic Analysis of Marinesco–Sjögren Syndrome Fibroblasts Indicates Pro-Survival Metabolic Adaptation to SIL1 Loss”. Potenza et al., 2021. DOI: 10.3390/ijms222212449.

It is believed that the loss of Sil1 protein, leading to disruption of ER proteostasis and a maladaptive UPR, plays a significant role in the pathogenic process of MSS, but the exact mechanisms of pathogenesis remain unclear. Furthermore, only specific tissues degenerate following Sil1 loss, while others display proteostatic alterations but without evident cellular damage. The lack of sufficient human *in vitro* models presents an obstacle to the investigation of rare neuromuscular diseases, but recent findings suggest that human skin fibroblasts, due to their ease of access, exhibit a protein signature that makes them a valuable tool for exploring disease etiology and the disrupted molecular pathways [40].

Based on this rationale, we decided to investigate the proteome of skin fibroblasts obtained from an MSS patient (HF-MSS) carrying the *SIL1* R111X mutation to identify the cellular mechanisms affected by the absence of Sil1 [21].

In this work, I contributed the most by performing the functional pathway analysis of proteomic data.

3.1.1 MSS Cellular Features in Cultured Skin Fibroblasts

Despite fibroblasts are not the primary cells affected by Sil1 loss, our analysis of HF-MSS revealed that these cells experience ER stress with a modest activation of UPR and exhibit increased autophagy. The truncated Sil1 was undetectable through Western blotting - likely due to its degradation - while higher levels of ATF4 and phosphorylated eIF2 α (at threonine 52), as well as an accumulation of the cleaved form of ATF6 were observed in comparison to healthy fibroblasts (HF-CTRL) (Figure 5A). The IRE1 pathway appeared inactive, as evidenced by unchanged sXBP1 levels assessed by PCR (Figure 5B). Additionally, among chaperones induced by ER stress, BiP and PDI (Protein disulfide isomerase) were upregulated in HF-MSS, while ORP150 didn't change (Figure 5C).

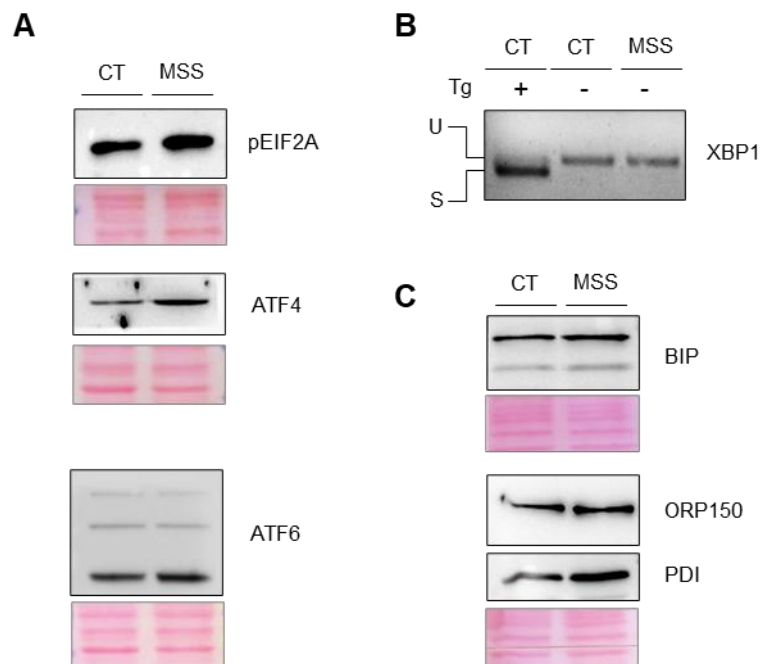


Figure 5 UPR activation in MSS patient fibroblasts. (A, C) Western blot analysis of HF-CTRL (CT) and HF-MSS (MSS) fibroblasts cultured under standard conditions showing expression of *SIL1*, ATF4, ATF6, and phosphorylated EIF2 α (A), and BiP, ORP150, and PDI (C). Ponceau S staining confirms equal protein loading. Blots shown are representative of at least two independent experiments. (B) XBP1 mRNA splicing was evaluated by PCR. HF-CTRL fibroblasts treated with 1 μ M thapsigargin (Tg) for 6 h served as a positive control. Unspliced (U) and spliced (S) XBP1 forms are indicated.
 From “Proteomic Analysis of Marinesco–Sjogren Syndrome Fibroblasts Indicates Pro-Survival Metabolic Adaptation to *SIL1* Loss”. Potenza et al., 2021. DOI: 10.3390/ijms222212449.

Morphologically, Sil1 loss results in an increase of autophagic vacuoles in cells of affected tissues, thus we investigated the presence of these structures in HF-MSS. In immunofluorescence, we found that the anti-lysosomal-associated membrane protein 1 (LAMP1) antibody detected a higher abundance of vacuoles in these cells (data not shown), a finding further supported by electron microscopy (performed by Laura Pietrangelo, University “G. d’Annunzio of Chieti-Pescara), which revealed the accumulation of multilamellar bodies and structures containing clustered membranes in HF-MSS compared to HF-CTRL (Figure 6A, 6B). Furthermore, we monitored cellular markers of autophagy in Western blotting, such as LAMP1, LC3-I/II and SQSTM1/p62 (sequestosome1) (Figure 6C). Both the elevated lipidated LC3-II levels, indicative of either increased autophagosome formation or impaired autophagic degradation, and the reduced SQSTM1/p62 levels, a receptor for ubiquitinated proteins degraded by autophagic vacuoles, suggested an enhancement of the autophagic degradation pathway.

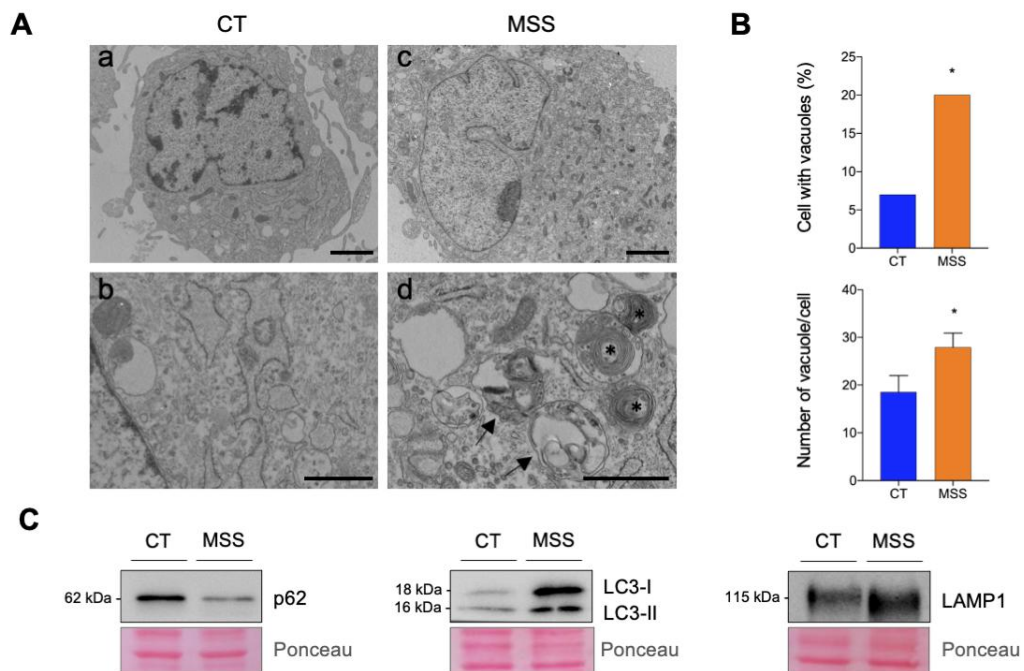


Figure 6 Electron microscopy (EM) and immunoblot analysis of autophagic vacuoles. (A) Ultrastructural alterations in HF-MSS. Representative EM images of HF-CTRL (a, b) and HF-MSS (c, d) fibroblasts. High-magnification views (b, d) show that HF-MSS cells accumulate vacuoles containing membrane clusters (arrowheads) and multilamellar bodies (asterisks). Scale bars: a, c = 2 μ m; b, d = 1 μ m. (B) Quantification of autophagic structures. Top graph: percentage of HF-CTRL and HF-MSS cells displaying vacuoles/multilamellar bodies ($n = 100$ cells/group). Bottom graph: average number of these structures per cell, considering only affected cells. Statistical analysis by Chi-square test (top) and unpaired t-test (bottom); * $p < 0.05$. (C) Western blot analysis of p62/SQSTM1, LC3, and LAMP1 in HF-CTRL (CT) and HF-MSS (MSS) fibroblasts under standard culture conditions. Ponceau S staining confirms equal protein loading. Immunoblots are representative of at least two independent experiments.

From "Proteomic Analysis of Marinesco-Sjogren Syndrome Fibroblasts Indicates Pro-Survival Metabolic Adaptation to *SIL1* Loss". Potenza et al., 2021. DOI: 10.3390/ijms222212449.

3.1.2 Proteomic Characterisation and Functional Pathways Analysis of HF-MSS

To understand the molecular changes and the adaptive mechanisms due to *Sil1* loss, thanks to the contribution of Damiana Pieragostino and other colleagues from our mass spectrometry facility, we performed shotgun proteomics of HF-MSS and control fibroblasts, which identified 625 differentially expressed (DE) proteins, with 305 significantly downregulated and 320 significantly upregulated (Figure 7A). We observed a strong Pearson correlation, with an R^2 value of 0.92, between the relative label-free quantification (LFQ) intensities of proteins in HF-MSS and HF-CT cells, showing excellent reproducibility in our measurements. Additionally, the protein density distribution along the mainline supported this high degree of reproducibility and indicated the absence of artefacts introduced during cell culture.

To analyse our dataset, we used several bioinformatic tools, such as STRING (<https://string->

[db.org](#)), Gene Ontology (GO) enrichment analysis, KEGG and Ingenuity Pathway Analysis (IPA, Qiagen, Hilden, Germany).

STRING revealed a robust protein-protein interaction (PPI) network, with 625 nodes and a highly significant enrichment ($p\text{-value} < 1.0 \times 10^{-16}$), even at the highest confidence level of PPI (0.9). Subnetworks of downregulated and upregulated proteins are shown in Figure 7B and Figure 7C, respectively.

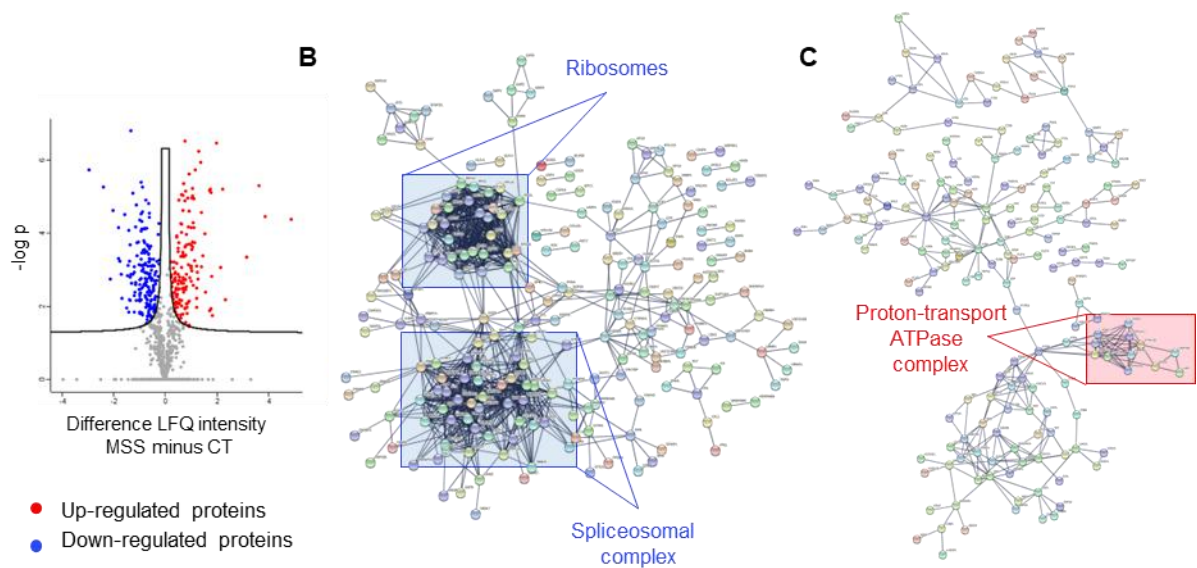


Figure 7 Proteomic analysis graphical networks. (A) Volcano plot showing differential protein expression between HF-CTRL and HF-MSS cells, generated using Perseus. LQF intensity of HF-MSS minus that of HF-CTRL was plotted against the negative logarithm of the p -values. (B) STRING interaction networks of significantly downregulated (left) and upregulated (right) proteins in HF-MSS cells, with a confidence score threshold of 0.9. Highlighted squares denote representative clusters of functionally related interacting proteins.

From “Proteomic Analysis of Marinesco–Sjogren Syndrome Fibroblasts Indicates Pro-Survival Metabolic Adaptation to *SIL1* Loss”. Potenza et al., 2021. DOI: 10.3390/ijms222212449.

GO analysis provided by STRING revealed several specific altered “Biological Processes”, with a significant number of downregulated proteins involved in mRNA metabolism, splicing and translation initiation, thus highlighting a reduction in overall protein synthesis. Conversely, we observed an enrichment of proteins involved in vesicle-mediated transport and metabolic pathways, with varying patterns of up- and downregulation (Table 1). Analysis of the “Molecular Functions” showed a general downregulation of proteins involved in RNA and cytoskeletal binding functions (Table 2), while “Cellular Components” enriched in DE proteins suggested significant changes in key organelles, like ER, vesicles and lysosomes (Table 3). Taken together, these data indicated that the absence of *Sil1* impacts negatively on protein synthesis but boosts degradation; however, complex protein functions made it difficult to draw

conclusions.

Term ID ¹	Term Description ²	Observed Gene Count ³	Background Gene Count ⁴	False Discovery Rate ⁵
GO:0016071	mRNA metabolic process	83	667	2.5×10^{-21}
GO:0000398	mRNA splicing, via spliceosome	45	284	6.16×10^{-15}
GO:0006397	mRNA processing	54	456	2.70×10^{-13}
GO:1903311	regulation of mRNA metabolic process	36	238	1.89×10^{-11}
GO:0043488	regulation of mRNA stability	21	113	4.54×10^{-8}
GO:0050684	regulation of mRNA processing	18	114	4.33×10^{-6}
GO:0048024	regulation of mRNA splicing, via spliceosome	15	77	4.38×10^{-6}
GO:0006413	translational initiation	30	142	1.17×10^{-12}
GO:0006412	translation	44	362	3.99×10^{-11}
GO:0006417	regulation of translation	38	327	3.73×10^{-9}
GO:0002181	cytoplasmic translation	15	57	1.90×10^{-7}
GO:0006446	regulation of translational initiation	8	71	0.0246
GO:0006614	SRP-dependent cotranslational protein targeting to membrane	23	92	6.11×10^{-11}
GO:0016192	vesicle-mediated transport	139	1699	6.02×10^{-21}
GO:0060627	regulation of vesicle-mediated transport	30	480	0.0077
GO:0006892	post-Golgi vesicle-mediated transport	9	83	0.0197
GO:0055114	oxidation-reduction process	94	932	4.04×10^{-9}
GO:0015980	energy derivation by oxidation of organic compounds	17	217	0.0112
GO:0044281	small molecule metabolic process	134	1779	1.21×10^{-17}
GO:0062012	regulation of small molecule metabolic process	21	332	0.0272

¹ Unique seven-digit identifier prefixed by GO. ² Textual description of the term. ³ Number of genes linked to that GO term in the input list. ⁴ Number of genes linked to a GO term in the entire background set. ⁵ Expected ratio of the number of false positive classifications (false discoveries) to the total number of positive classifications.

Table 1 Selected biological processes altered in HF-MSS, based on Gene Ontology enrichment analysis.

From “Proteomic Analysis of Marinesco–Sjogren Syndrome Fibroblasts Indicates Pro-Survival Metabolic Adaptation to *SIL1* Loss”. Potenza et al., 2021. DOI: 10.3390/ijms222212449.

Term ID ¹	Term Description ²	Observed Gene Count ³	Background Gene Count ⁴	False Discovery Rate ⁵
GO:0003723	RNA binding	86	850	2.57×10^{-17}
GO:0003729	mRNA binding	36	198	2.76×10^{-13}
GO:0003727	single-stranded RNA binding	13	80	0.00011
GO:0003725	double-stranded RNA binding	11	70	0.00058
GO:0019843	rRNA binding	10	60	0.00079
GO:0008135	translation factor activity, RNA binding	11	84	0.0021
GO:0000049	tRNA binding	8	56	0.0079
GO:0061980	regulatory RNA binding	5	34	0.0424
GO:0016491	oxidoreductase activity	75	716	8.70×10^{-16}
GO:0016860	intramolecular oxidoreductase activity	12	47	5.01×10^{-6}
GO:0000166	nucleotide binding	134	2097	2.45×10^{-12}
GO:0017076	purine nucleotide binding	108	1865	1.36×10^{-7}
GO:0030554	adenyl nucleotide binding	79	1524	0.00039
GO:0008092	cytoskeletal protein binding	71	882	4.82×10^{-10}
GO:0046961	proton-transporting ATPase activity, rotational mechanism	9	21	4.97×10^{-6}
GO:0008553	proton-exporting ATPase activity, phosphorylative mechanism	9	23	8.55×10^{-6}
GO:0015078	proton transmembrane transporter activity	12	120	0.0082
GO:0005178	integrin binding	19	122	2.30×10^{-6}
GO:0050839	cell adhesion molecule binding	24	200	3.68×10^{-6}
GO:0098634	cell-matrix adhesion mediator activity	3	7	0.0229

¹ Unique seven-digit identifier prefixed by GO. ² Textual description of the term. ³ Number of genes linked to that GO term in the input list. ⁴ Number of genes linked to a GO term in the entire background set. ⁵ Expected ratio of the number of false positive classifications (false discoveries) to the total number of positive classifications.

Table 2 Selected molecular functions altered in HF-MSS, based on Gene Ontology enrichment analysis.

From “Proteomic Analysis of Marinesco–Sjogren Syndrome Fibroblasts Indicates Pro-Survival Metabolic Adaptation to *SIL1* Loss”. Potenza et al., 2021. DOI: 10.3390/ijms222212449.

Term ID ¹	Term Description ²	Observed Gene Count ³	Background Gene Count ⁴	False Discovery Rate ⁵
GO:0043226	organelle	566	12432	1.37×10^{-50}
GO:0043229	intracellular organelle	560	12193	3.30×10^{-50}
GO:0043227	membrane-bounded organelle	516	11244	1.07×10^{-37}
GO:0005783	endoplasmic reticulum	120	1796	9.40×10^{-13}
GO:0005788	endoplasmic reticulum lumen	30	299	1.86×10^{-6}
GO:0005793	endoplasmic reticulum-Golgi intermediate compartment	13	113	0.00095
GO:0031982	vesicle	173	2318	4.41×10^{-24}
GO:0031410	cytoplasmic vesicle	166	2226	6.49×10^{-23}
GO:0099503	secretory vesicle	96	948	1.62×10^{-20}
GO:0030139	endocytic vesicle	35	275	8.75×10^{-10}
GO:0045335	phagocytic vesicle	23	122	2.25×10^{-9}
GO:0042470	melanosome	35	105	1.55×10^{-20}
GO:1990904	ribonucleoprotein complex	89	770	2.33×10^{-22}
GO:0030532	small nuclear ribonucleoprotein complex	11	67	0.00021
GO:0005773	vacuole	74	682	4.05×10^{-17}
GO:0005775	vacuolar lumen	30	172	2.47×10^{-11}
GO:0005774	vacuolar membrane	31	311	1.36×10^{-6}
GO:0005764	lysosome	67	582	1.24×10^{-16}
GO:0015629	actin cytoskeleton	42	432	1.75×10^{-8}
GO:0032432	actin filament bundle	12	57	1.38×10^{-5}

¹ Unique seven-digit identifier prefixed by GO. ² Textual description of the term. ³ Number of genes linked to that GO term in the input list. ⁴ Number of genes linked to a GO term in the entire background set. ⁵ Expected ratio of the number of false positive classifications (false discoveries) to the total number of positive classifications.

Table 3 Selected cellular components altered in HF-MSS, based on Gene Ontology enrichment analysis. From “Proteomic Analysis of Marinesco–Sjogren Syndrome Fibroblasts Indicates Pro-Survival Metabolic Adaptation to *SIL1* Loss”. Potenza et al., 2021. DOI: 10.3390/ijms222212449.

In order to get a clearer understanding of the molecular processes involved, we used the KEGG database (Table 4). Pathways enrichment analysis of HF-MSS exhibited a reduction of the protein synthesis, with downregulation of proteins related to spliceosome, RNA transport and ribosomes, but also an upregulation of phagosome and lysosome pathways, suggesting an increased protein degradation through these ways. Interestingly, cellular metabolism showed significant alterations, resulting in enhanced energy production - upregulated fatty acid catabolism, TCA cycle, amino acids/glycan/glycosaminoglycan degradation pathways – and a reduction in biosynthesis – downregulated fatty acid synthase, arginine/serine/glycine/cysteine biosynthetic pathways. Conversely, some cholesterol biosynthetic enzymes were upregulated, thus possibly indicating cholesterol involvement in the membrane formation of vacuoles and multilamellar bodies. Glycolysis/gluconeogenesis and pentose phosphate pathways showed mixed regulation, so it was impossible to predict their activation or inhibition. To summarise, HF-MSS cells undergo deep metabolic changes to fulfil cellular needs in response to ER stress.

Term ID ¹	Term Description ²	Observed Gene Count ³	Background Gene Count ⁴	False Discovery Rate ⁵
hsa03040	spliceosome	26	130	3.14×10^{-10}
hsa03010	ribosome	21	130	3.48×10^{-7}
hsa03013	RNA transport	14	159	0.0074
hsa04145	phagosome	26	145	1.35×10^{-9}
hsa04142	lysosome	24	123	1.39×10^{-9}
hsa01100	metabolic pathways	99	1250	1.52×10^{-13}
hsa00020	citrate cycle (TCA cycle)	9	30	7.00×10^{-5}
hsa01212	fatty acid metabolism	10	48	0.00024
hsa01230	biosynthesis of amino acids	11	72	0.00075
hsa00010	glycolysis/Gluconeogenesis	10	68	0.0017
hsa00030	pentose phosphate pathway	5	30	0.0259

¹ Unique identifier for each KEGG object which takes the form of a prefix followed a five-digit number. ² Textual description of the term.

³ Number of genes linked to that KEGG term in the input list. ⁴ Number of genes linked to a KEGG term in the entire background set.

⁵ Expected ratio of the number of false positive classifications (false discoveries) to the total number of positive classifications.

Table 4 Selected altered pathways in HF-MSS, based on KEGG enrichment analysis.

From “Proteomic Analysis of Marinesco–Sjogren Syndrome Fibroblasts Indicates Pro-Survival Metabolic Adaptation to *SIL1* Loss”. Potenza et al., 2021. DOI: 10.3390/ijms222212449.

By using IPA, we identified 170 significantly affected “Canonical Pathways”, thus confirming a general involvement of all cellular compartments (Table 5). In accordance with KEGG, IPA confirmed alterations of the phagosome, beta-oxidation of fatty acids, TCA cycle, and degradation of amino acids. It also highlighted mitochondrial dysfunction and eIF2 signalling inhibition, aligning with PERK activation.

Finally, among the predicted “Disease and Functions”, in HF-MSS we found increased degradation and metabolic activities, along with a decrease in DNA repair and cell cycle progression (not significant). Notably, IPA suggested a decrease in movement disorders and neurodegeneration, but this was misleading due to upregulated lysosomal enzymes (Table 6).

Ingenuity Canonical Pathway ¹	–log (p-Value) ²	z-Score ³
phagosome maturation	14.6	
EIF2 signaling	14	–2.357
caveolar-mediated endocytosis signaling	7.97	
fatty acid β -oxidation I	7.88	2.53
TCA cycle II (Eukaryotic)	6.89	2.121
isoleucine degradation I	6.83	2.236
mitochondrial dysfunction	6.78	
valine degradation I	4.99	2.236
tryptophan degradation III (Eukaryotic)	4.49	1.342
spliceosomal cycle	3.51	–2.646
tRNA charging	2.43	–1.342

¹ Textual description of the term. ² Negative logarithm of p-value. ³ Also called standard score, is the number of standard deviations by which the value of a raw score is above or below the mean value of what is being observed or measured.

Table 5 Selected canonical pathways altered in HF-MSS, based on Ingenuity Pathway Analysis.

From “Proteomic Analysis of Marinesco–Sjogren Syndrome Fibroblasts Indicates Pro-Survival Metabolic Adaptation to *SIL1* Loss”. Potenza et al., 2021. DOI: 10.3390/ijms222212449.

Disease or Functions Annotation ¹	p-Value	z-Score ²	Predicted Activation State ³
fatty acid metabolism	4.57×10^{-12}	2.017	Increased
cellular homeostasis	5.29×10^{-11}	2.129	Increased
cell death of tumor cell lines	8.41×10^{-24}	2.15	Increased
endocytosis by eukaryotic cells	5.24×10^{-11}	2.311	Increased
metabolism of glycosphingolipid	0.00000134	2.387	Increased
cell death of tumor cells	1.13×10^{-13}	2.416	Increased
sensitivity of cells	0.00000386	2.56	Increased
phagocytosis	3.93×10^{-8}	2.708	Increased
phagocytosis of cells	0.00000438	2.714	Increased
degradation of extracellular matrix	7.31×10^{-8}	2.905	Increased
cell death of cancer cells	1.17×10^{-10}	2.995	Increased
cell death of osteosarcoma cells	1.76×10^{-12}	3.545	Increased
homologous recombination	9.48×10^{-9}	-3.637	Decreased
accumulation of lipid	2.49×10^{-9}	-3.636	Decreased
homologous recombination of cells	5.85×10^{-9}	-3.499	Decreased
glucose metabolism disorder	0.00000102	-3.279	Decreased
DNA recombination	6.51×10^{-9}	-3.235	Decreased
motor dysfunction or movement disorder	1.01×10^{-18}	-2.934	Decreased
movement Disorders	9.11×10^{-19}	-2.666	Decreased
lysosomal storage disease	2.68×10^{-10}	-2.591	Decreased
repair of DNA	0.00000166	-2.545	Decreased
cell death of breast cancer cell lines	9.12×10^{-8}	-2.374	Decreased
astrocytosis	0.00000147	-2.219	Decreased
degeneration of central nervous system	8.66×10^{-9}	-2.218	Decreased
neurodegeneration	0.00000465	-2.185	Decreased
degeneration of nervous system	0.00000827	-2.128	Decreased
amyloidosis	7.3×10^{-13}	-2.08	Decreased
degeneration of brain	1.46×10^{-8}	-2.032	Decreased

¹ Textual description of the term. ² Also called standard score, is the number of standard deviations by which the value of a raw score is above or below the mean value of what is being observed or measured. ³ Prediction based on the observed gene expression changes in the dataset.

Table 6 Selected diseases or functions that are predicted to be increased or decreased on the basis of differential proteins in HF-MSS, based on Ingenuity Pathway Analysis.

From “Proteomic Analysis of Marinesco–Sjogren Syndrome Fibroblasts Indicates Pro-Survival Metabolic Adaptation to *SIL1* Loss”. Potenza et al., 2021. DOI: 10.3390/ijms222212449.

3.1.3 Proteomic Data Validation

To confirm *in vitro* some molecular modifications evidenced by proteomics, we decided to analyse the expression of selected proteins. Specifically, in HF-MSS fluorescence microscopy revealed a significant reduction in nucleolin (NCL) and nucleophosmin (NPM), both crucial for ribosome biogenesis, which aligned with the proteomic identification of downregulated RNA processing and translation. In addition, we found increased levels of calnexin and PDI – upregulated chaperones identified by proteomics - within the enlarged ER cisternae of these cells, which were notably larger than controls (data not shown).

We performed western blotting analysis to provide additional validation of the proteomic findings, confirming reduced levels of NCL, NPM and nestin (NES), and the upregulation of caveolin-1 (CAV-1), annexin-V (ANXA-5) and collagen triple helix repeat containing 1 (CTHRC1) (Figure 8).

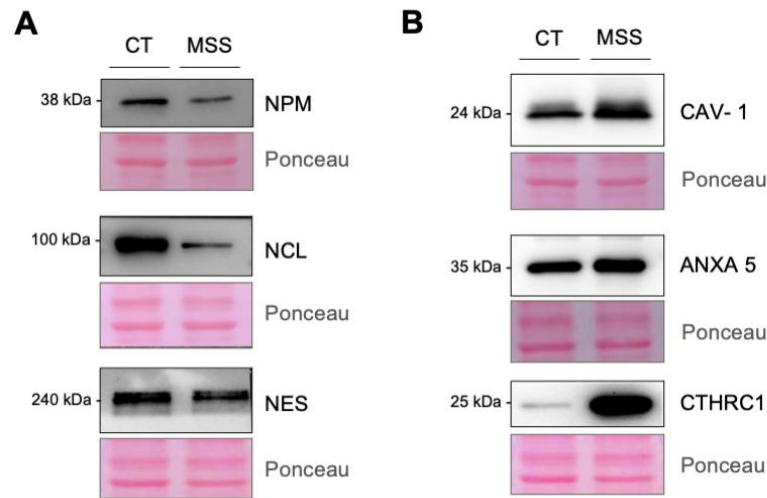


Figure 8 Western blot validation of proteomic analysis. (A, B) Western blot analysis of HF-CTRL (CT) and HF-MSS (MSS) fibroblasts cultured under standard conditions. Downregulated proteins in HF-MSS (NPM, NCL, NES) are shown in (A), while upregulated proteins (CAV-1, ANXA-5, CTHRC1) are shown in (B). Ponceau S staining confirms equal protein loading. Immunoblots are representative of at least two independent experiments. From “Proteomic Analysis of Marinesco–Sjogren Syndrome Fibroblasts Indicates Pro-Survival Metabolic Adaptation to *SIL1* Loss”. Potenza et al., 2021. DOI: 10.3390/ijms222212449.

Finally, given the strong downregulation of ribosome and RNA splicing components, as revealed by our proteomics analysis and further supported by the IPA 'Disease or Functions' predictions, we monitored the cell growth rate of HF-MSS cells. The incorporation of EdU, a thymidine analogue, revealed a significant reduction in cell proliferation compared to controls (data not shown).

In summary, our data suggested that MSS fibroblasts exhibit morphological and biochemical signs of activated UPR and deep metabolic alterations to cope with the absence of Sil1 loss.

3.2 Transcriptomic Analysis of Marinesco-Sjögren Syndrome Fibroblasts

From “Sil1-deficient fibroblasts generate an aberrant extracellular matrix leading to tendon disorganisation in Marinesco-Sjögren syndrome” Amodei et al., 2024. DOI: 10.1186/s12967-024-05582-0.

Prompted by the insights derived from our proteomics study and in order to get a more detailed perspective on the molecular dynamics involved in the pathogenesis of MSS, we decided to explore the transcriptome of HF-MSS [41]. Through transcriptomic analysis, it is possible to identify the complete set of RNA transcripts, effectively mirroring gene activity in response to disease. Moreover, it offers the opportunity to discover novel biomarkers, thereby facilitating the development of new diagnostic and therapeutic strategies.

In this work, in which I contributed equally as first author, I was mainly responsible for the functional analysis of pathways and the execution of cellular experiments.

3.2.1 Transcriptomic Characterisation and Functional Pathways Analysis of MSS Fibroblasts

To investigate the gene expression profile of MSS fibroblasts, we conducted a transcriptomic analysis in biological triplicate on skin fibroblasts from the same patient carrying the *SIL1* R111X mutation (HF-MSS).

For total RNA extraction and subsequent next-generation sequencing, cells were cultured under standard conditions, and healthy fibroblasts were used as controls (HF-CTRL).

We identified 664 DE transcripts with a fold change (FC) > 2 and FDR < 0.1, with the top 50 most variable genes shown in the heat map (Figure 9A).

Following the methodology employed in our previous proteomics study, we queried the STRING PPI database, which revealed a significantly enriched network of interactions among DE transcripts ($p < 6.7 \times 10^{-12}$), supporting the robustness of our findings.

Additionally, from our gene set, GO enrichment analysis identified 237 biological processes, 6 molecular functions, 30 cellular components and 24 subcellular compartments. These results highlighted a membrane trafficking issue in HF-MSS, in line with previous studies [32].

Interestingly, a significant number of DE genes were associated with intracellular organelles (more than 350) and extracellular matrix (ECM)-related compartments (more than 150), and the cellular components enrichment also confirmed the involvement of both endomembrane system and ECM/cell adhesion mechanisms (Figure 9B).

To evaluate whether our identified gene alterations were indicative of MSS, we decided to

compare the enriched biological processes of DE genes with those of our previous proteomic study on the same fibroblasts [21] and with those of *Sil1^{Gt}* mouse muscle [25].

STRING analysis revealed an overlap of about 50% between the current transcriptomics and our proteomics, while *Sil1^{Gt}* mouse muscle proteomics shared about 30% of biological processes. These levels of overlap are very significant, as demonstrated by a computer simulation that showed only 3.2% overlap when using 898 randomly selected biological processes.

To further explore the functional implications of the DE genes, we used IPA. Among the “Canonical Pathways”, in HF-MSS IPA identified the downregulation of the glycoprotein VI(GP6) signalling and the glioma invasiveness signalling, along with the tumour microenvironment, the signalling by Rho family GTPases, the pulmonary fibrosis idiopathic signalling and the hepatic fibrosis signalling.

Moreover, through the “Disease and Functions” IPA module, we found out the involvement of ECM alterations (decreased cellular movement of tumor cell line/migration of tumor cell line) and increased cell death with reduced proliferation (increased apoptosis; decreased cell survival/cell proliferation of tumour cell lines), in line with our previous study [21].

To provide an experimental validation of our transcriptomic analysis, we performed quantitative real-time PCR (qPCR) on 13 DE genes, including *SIL1*. These genes were selected for their higher FC and lower FDR, and also for their relevance to the functional alterations associated with MSS (ECM remodelling, cell cytoskeleton dynamics, vesicle trafficking, endo-lysosome autophagy pathways).

The qPCR of the reverse-transcribed total RNA extracted from cells confirmed the upregulation of seven genes and the downregulation of five genes in HF-MSS (Figure 9C).

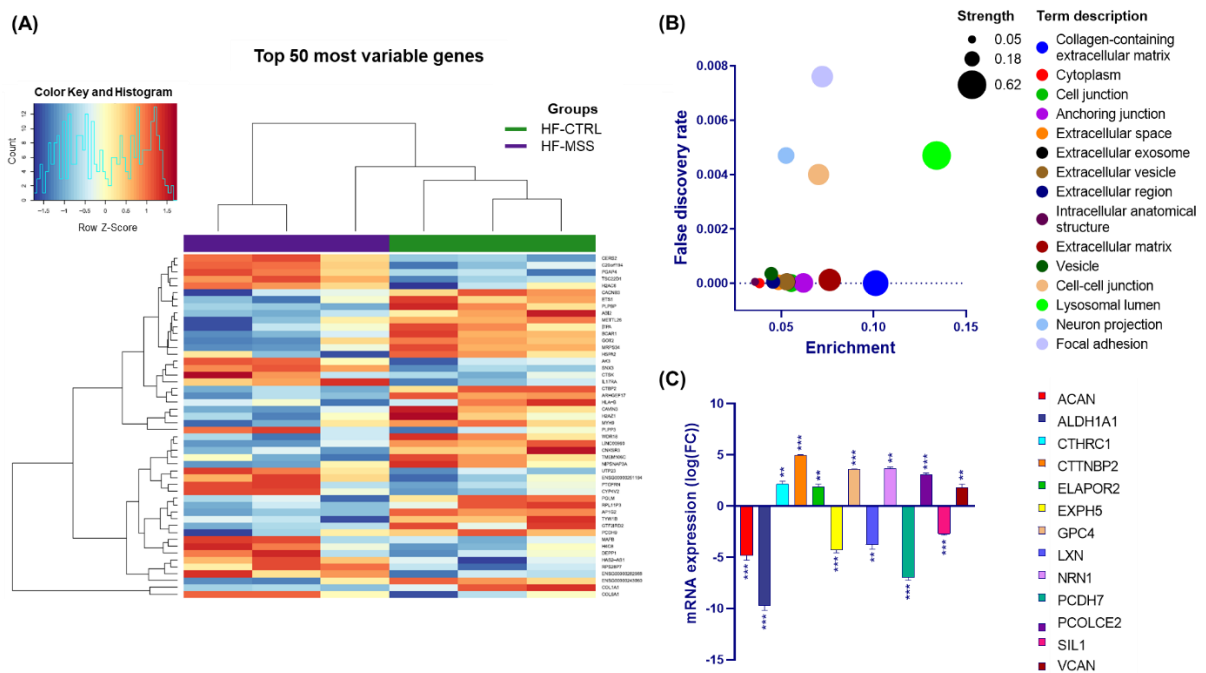


Figure 9 Differentially expressed genes in fibroblasts from an MSS patient compared with a healthy control. (A) Heatmap displaying the top 50 most variable genes based on log fold change (logFC). Upregulated genes are shown in red tones, and downregulated genes in blue, according to the colour key. Control and patient fibroblast samples are marked by green and violet bars, respectively. Gene names are listed on the right. (B) Bubble plot representing enriched cellular component gene ontologies among differentially expressed genes. Enrichment is expressed as the ratio of observed to expected genes per ontology. Bubble size reflects the enrichment ratio, and colour intensity indicates the enrichment strength (Log10 of the observed-to-expected gene ratio). (C) Validation of transcriptomic data by quantitative RT-PCR for 12 selected genes. Data are shown as fold change (log scale), with gene names indicated in the legend. Results represent mean $\pm$ standard error from three independent experiments performed in technical triplicate. Statistical analysis of Δ Ct values was performed using one-way ANOVA (** $p < 0.01$, *** $p < 0.001$). From “Sil1-deficient fibroblasts generate an aberrant extracellular matrix leading to tendon disorganisation in Marinesco-Sjögren syndrome” Amodei et al., 2024. DOI: 10.1186/s12967-024-05582-0.

3.2.2 Functional Validation of ECM-Related Genes Alterations in MSS Fibroblasts

To determine the functional impact of the DE genes related to ECM and anchoring structures, we decided to perform several biochemical experiments on HF-MSS cells.

Firstly, we focused on the study of ECM remodelling, a process by which metalloproteases, like collagenases, cleave proteins for internalisation and lysosomal hydrolysis. For this purpose, we use a fluorescent dye-quenched (DQ)-collagen probe able to emit fluorescence only upon digestion by collagenases. Time course experiments revealed fluorescence dots in both control and patient fibroblasts after 3 hours of incubation with DQ-collagen, but only control cells showed increased fluorescence over time (Figure 10A and B).

In addition, confocal microscopy showed that intracellular DQ-collagen dots colocalised with

the lysosomal marker “LysoTracker” in both cells, but HF-MSS exhibited a marked reduction in DQ-collagen internalisation and increased surrounding fluorescent filaments, thus suggesting slowed ECM metabolism (Figure 10C and D and F).

As additional evidence supporting the reduced metalloprotease activity, we measured DQ-collagen digestion in conditioned medium, which was lower in patient cells (Figure 10E).

These findings confirmed the molecular changes impacting the ECM remodelling identified by transcriptomics.

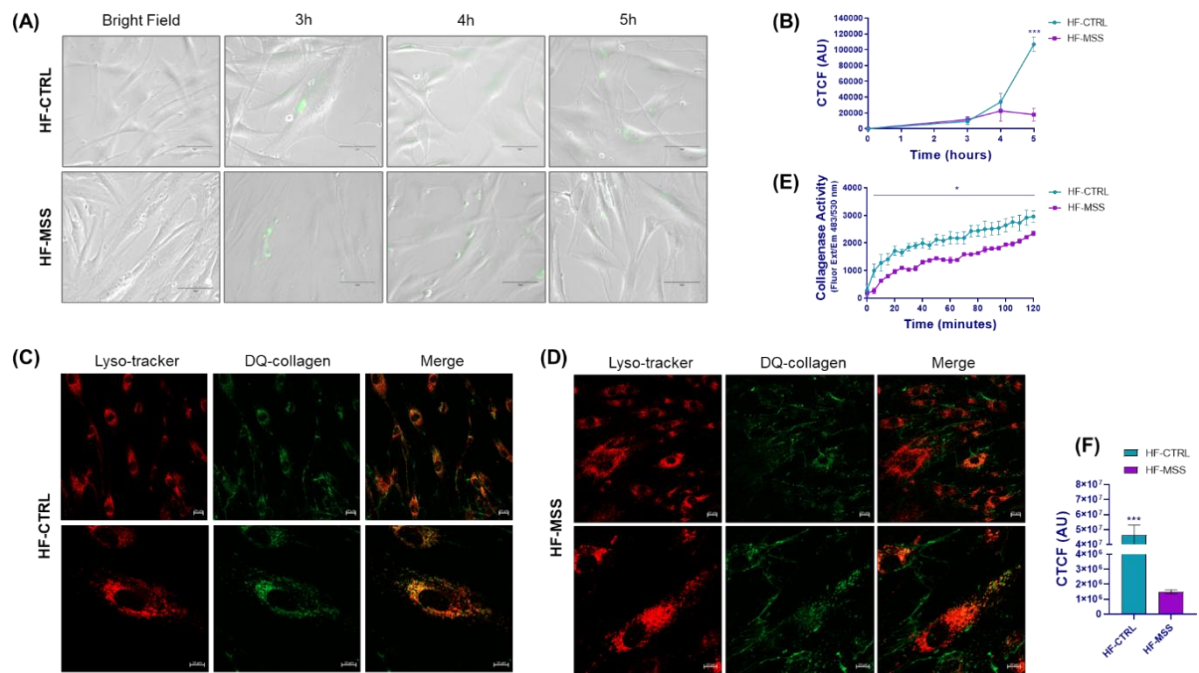


Figure 10 Live cell imaging of DQ-collagen internalisation in fibroblasts and collagenase activity.

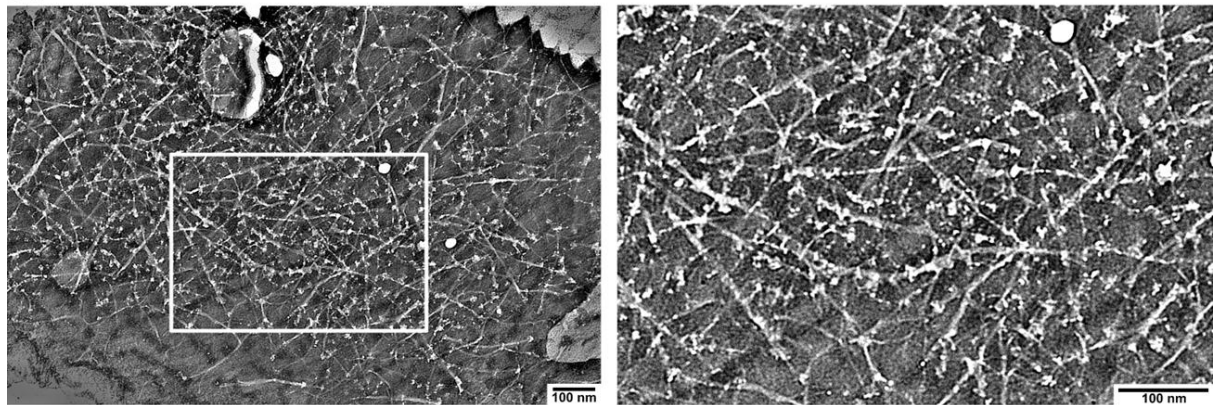
(A) Representative images showing time-dependent internalisation of DQ-collagen in control (HF-CTRL) and patient (HF-MSS) fibroblasts. (B) Quantification of DQ-collagen fluorescence from (A), expressed as corrected total cell fluorescence (CTCF) in arbitrary units. Data are presented as mean $\pm$ standard error from three independent experiments. Statistical analysis by repeated measures ANOVA with post-hoc multiple t-tests; *** $p < 0.001$, $q < 0.001$. (C–D) Confocal images of live HF-CTRL (C) and HF-MSS (D) fibroblasts incubated with DQ-collagen for 5 h followed by LysoTracker for 3 h. Green and red channels represent DQ-collagen and LysoTracker fluorescence, respectively; merged images show colocalisation. Two magnifications are shown. Results are representative of two independent experiments performed in technical triplicate. (E) Collagenase activity assay of fibroblast-conditioned media. Cell-free supernatants were incubated with DQ-collagen, and fluorescence was measured every 5 minutes. Data are shown as mean $\pm$ standard error from two independent experiments in technical triplicate. Repeated measures ANOVA with post-hoc t-tests; * $p < 0.05$. (F) Quantification of internalised collagen in HF-CTRL and HF-MSS cells from (C–D); *** $p < 0.001$.

From “*Sil1-deficient fibroblasts generate an aberrant extracellular matrix leading to tendon disorganisation in Marinesco-Sjögren syndrome*” Amodei et al., 2024. DOI: 10.1186/s12967-024-05582-0.

With the intention of directly visualising potential structural modifications in the ECM, we collected the conditioned medium in which both HF-MSS and HF-CTRL had released ECM

components. With the contribution of Matteo Ardini from the University of L'Aquila, who performed the transmission electron microscope (TEM) analysis, we found out that control fibroblasts secreted a network of thin (11 nm), uniform protein filaments (Figure 11A). Conversely, HF-MSS also produced a network, but filaments were thicker (48.8 nm), ribbon-shaped, and tended to aggregate into structures resembling early-collagen fibrils (Figure 11B). These findings led us to hypothesise that at 24 hours of deposition, the ECM of HF-CTRL fibroblasts was still developing, whereas the patient cells, due to their impaired ECM remodeling capabilities, Thus, a possible explanation is that while HF-CTRL presented an immature ECM at 24 h of deposition, patient cells formed larger but disorganised fibrillar aggregates as a consequence of impaired ECM remodelling.

(A) HF-CTRL



(B) HF-MSS

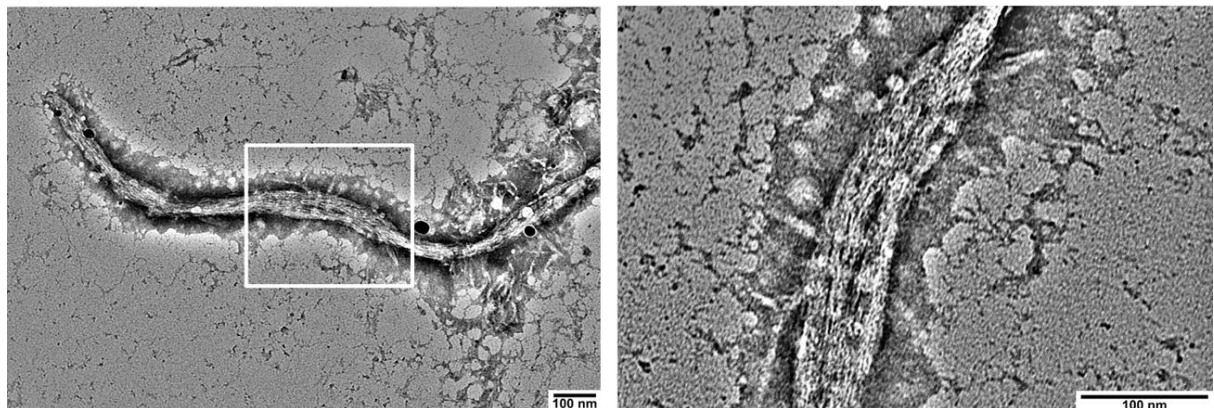


Figure 11 TEM analysis of negative-stained ECM released by fibroblasts. Fibroblasts were cultured to 80% confluence and incubated in serum-free HBSS medium for 24 hours. Conditioned medium was collected, and 5 μ L drops were applied to carbon-coated grids, stained, and air-dried prior to transmission electron microscopy (TEM). (A) Representative TEM images of ECM released by control fibroblasts (HF-CTRL) at low (left) and high (right) magnification. (B) Representative TEM images of ECM released by MSS patient fibroblasts (HF-MSS) at low (left) and high (right) magnification. Results are representative of two independent experiments performed in technical triplicate.

From “*Sil1-deficient fibroblasts generate an aberrant extracellular matrix leading to tendon disorganisation in Marinesco-Sjögren syndrome*” Amodei et al., 2024. DOI: 10.1186/s12967-024-05582-0.

To further validate the presence of alterations in ECM, cytoskeleton and anchor junctions revealed by transcriptomic data, we investigated the functional consequences on cell adhesion and motility. Firstly, we found that HF-MSS fibroblasts adhered to and spread on Petri dishes at a slower rate compared to controls, possibly due to the aberrant ECM fibrillar structures and altered adhesion receptors expression (Figure 12A and B)

In addition, the wound-healing assay revealed reduced motility in patient cells. Specifically, by monitoring for 24 h the closure of scratches made in confluent HF-MSS and HF-CTRL cells, and analysing the data with Scratch Wound Analysis Software (Sartorius), we found a significantly slower rate of movement in the diseased fibroblasts (Figure 12C and D).

However, while changes in ECM could be at the basis of these phenomena, the contribution of intrinsic cellular factors should be taken into account.

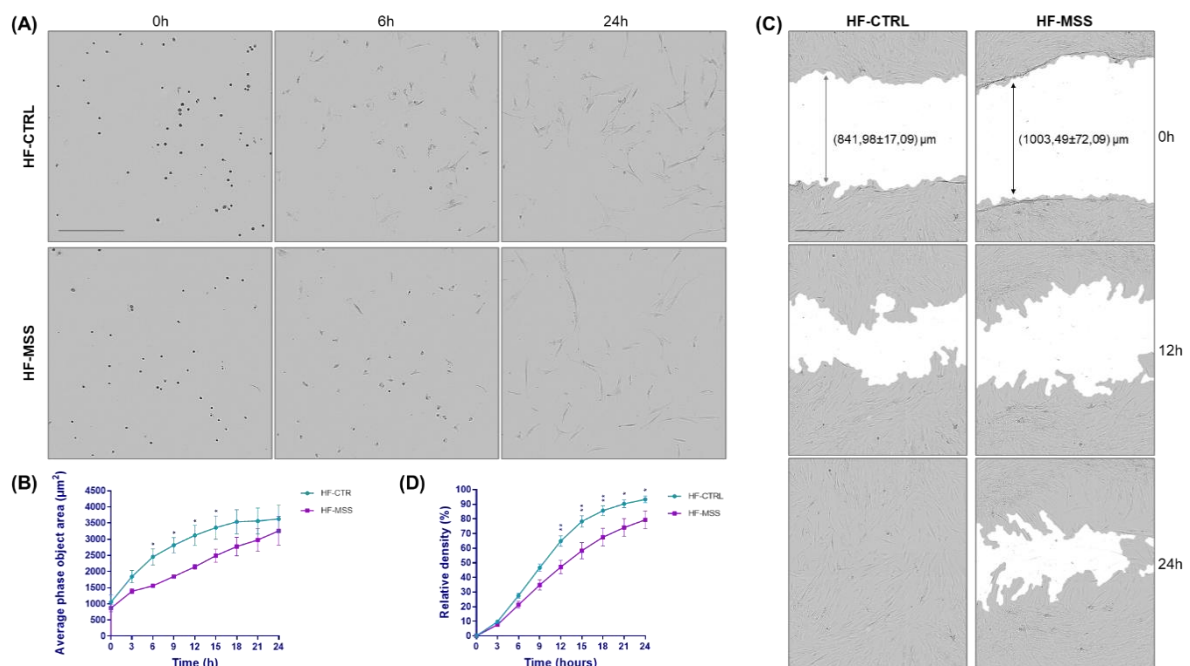


Figure 12 Analysis of fibroblast-ECM interactions. (A) Representative images showing fibroblast attachment over time. Control (HF-CTRL) and patient (HF-MSS) fibroblasts were seeded in 96-well plates and imaged at 0, 6, and 24 hours. Differences in attachment are evident as early as 6 hours. Scale bar: 400 μm. (B) Quantification of cell-covered area over time. Data are presented as mean ± standard error from three independent experiments performed in technical quadruplicate. Statistical analysis by repeated measures ANOVA with post-hoc multiple t-tests; * $p < 0.05$. (C) Wound-healing assay. Confluent monolayers of HF-CTRL and HF-MSS fibroblasts were scratched and imaged every 3 hours for 24 hours to monitor wound closure. Images at 0, 12, and 24 hours illustrate impaired migration in HF-MSS. Scale bar: 400 μm. (D) Quantitative analysis of cell migration shown in (C), expressed as relative wound density. Data represent mean ± standard error from four independent experiments performed in technical quadruplicate. Statistical analysis by repeated measures ANOVA with post-hoc multiple t-tests; * $p < 0.05$, ** $p < 0.01$.

From "Sil1-deficient fibroblasts generate an aberrant extracellular matrix leading to tendon disorganisation in Marinesco-Sjögren syndrome" Amodei et al., 2024. DOI: 10.1186/s12967-024-05582-0.

3.2.3 *In Vivo Evidence of Disrupted ECM Organisation in The Woozy Mouse Model of Marinesco-Sjögren Syndrome*

Given the marked abnormalities in the ECM displayed by MSS fibroblasts, we decided to examine whether an *in vivo* animal model also exhibited issues with ECM deposition and remodelling. Using TEM and with the collaboration of Laura Pietrangelo, we analysed the interface between the Achilles tendon and soleus muscle in both woozy mice and controls at seven months of age.

In semi-thin sections stained with toluidine blue, healthy tendons displayed the regular striated pattern of collagen fibrils (in dark blue) at the tendon-muscle boundary (Figure 13A). On the other hand, the connective tissue of woozy mouse appeared disorganised and characterized by less compact fibers (irregular blue staining) (Figure 13C).

Muscle-tendon junctions (MTJ) were identified in both samples (asterisks in Figure 13A and 13C), but at TEM higher magnifications it was possible to compare the morphology of tenocytes, the specific fibroblasts of this region. Surprisingly, while these cells appeared oblong and with a regular subcellular compartment in controls, in wz mice they exhibited a round shape and degenerative alterations, such as a larger nucleus, damaged mitochondria and cell debris (Figure 13B and D).

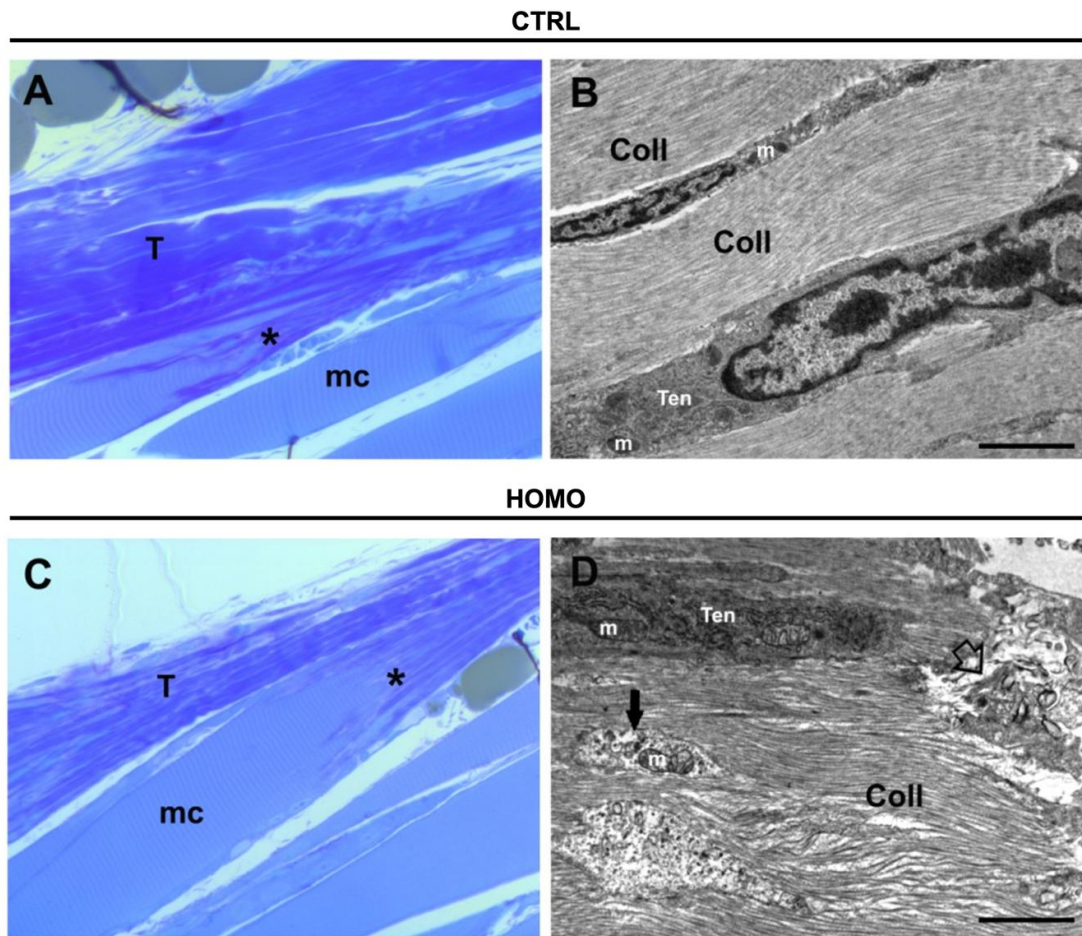


Figure 13 Light and transmission electron microscopy (TEM) analysis of Achilles tendons of control and woozy mouse. (A, C) Semi-thin sections stained with toluidine blue showing the boundary between the Achilles tendon (T) and soleus muscle (mc) in heterozygous control (*SIL1*^{+/-}, CTRL; A) and homozygous woozy (*SIL1*^{-/-}, HOMO; C) mice. The myotendinous junction (MTJ) is marked by an asterisk. (B, D) Transmission electron microscopy (TEM) images at higher magnification depicting the connective tissue of control (CTRL; B) and woozy (HOMO; D) mice. Tenocytes (Ten) are seen embedded between extracellular matrix collagen fibrils (Coll). Visible features include mitochondria (m), cell debris (black arrow), and regions of degeneration (empty arrow).

From “*Sil1*-deficient fibroblasts generate an aberrant extracellular matrix leading to tendon disorganisation in Marinesco-Sjögren syndrome” Amodei et al., 2024. DOI: 10.1186/s12967-024-05582-0.

In summary, these findings in the woozy mouse model supported our *in vitro* results, suggesting that defective ECM deposition and remodelling, as indicated by the abnormal collagen organisation and tenocytes morphology, are common features of MSS across species. Furthermore, given the critical role of the ECM, its altered structure may contribute to the impairment of muscles and other tissues in MSS.

3.3 Molecular Characterisation of A Newly Identified Pathogenic Variant in The *SIL1* Gene Causing Marinesco-Sjögren Syndrome

From “Structural and functional analysis of the novel pathological variant c.1024G>A in the *SIL1* gene causing Marinesco-Sjögren syndrome” Ruggieri et al., in submission. UNPUBLISHED DATA

In view of the existing scientific understanding and our recent contributions to the knowledge of MSS molecular mechanisms, we dedicated our efforts to the study of a new variant of the *SIL1* gene. To date, over 300 variants in the *SIL1* gene have been reported, but only a minority is identified as pathogenic, with a considerable number classified as variants of uncertain significance (VUS) (<https://clinvarminer.genetics.utah.edu/variants-by-gene/SIL1>).

In vitro studies are crucial for determining the pathogenicity of newly identified *SIL1* variants and aiding in the differential diagnosis of MSS. Thus, we were engaged by a Greek genetics research group to perform a deep molecular characterisation of a novel VUS in the *SIL1* gene identified in a pediatric patient with MSS-like symptoms, aiming to determine its pathogenic nature.

Periklis Makrythanasis and his colleagues from the University of Athens evaluated the clinical picture of this child, who presented hypotonia and developmental delay by age one, and later also significant speech and motor impairments. Clinical findings included astigmatism, hypermetropia and esotropia with normal audiological and cardiological evaluations; in addition, brain MRI showed cerebellar atrophy. At the age of three, electromyography suggested myopathic changes, and a global developmental delay was confirmed with severe limitations in communication, motor skills, and cognitive functions. At the most recent follow-up at 4 years old, the little patient showed minimal motor improvement but had developed some speech, thanks to occupational and speech therapy. Through whole-exome sequencing (WES) the Greek group identified the new homozygous missense variant NM_022464.5:c.1024G>A in the *SIL1* gene, confirmed by Sanger sequencing of the proband and her parents, who were found to be heterozygous carriers (Figure 14). According to the guidelines established by the American College of Medical Genetics (ACMG), this variant was classified primarily as a VUS. This classification highlighted the need for additional studies to definitively assess its pathogenicity through a molecular diagnosis, in which we were primarily involved.

I personally played a key role in this project, specifically by conducting most of the *in vitro* experiments necessary to assess the pathogenic nature of the VUS.

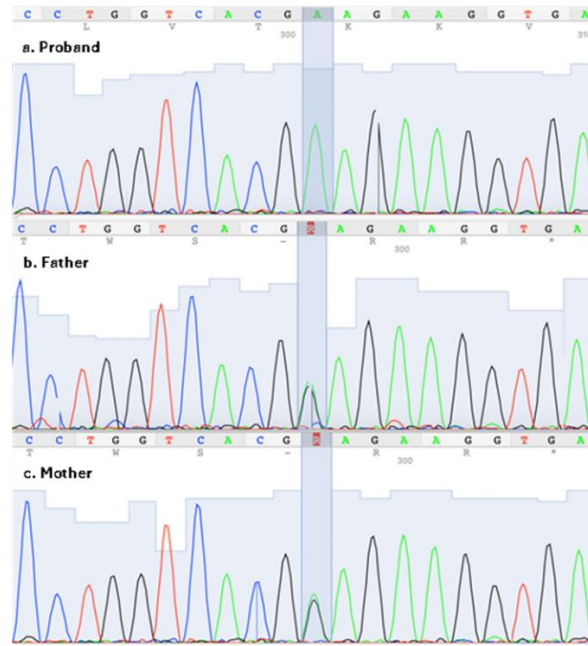


Figure 14 Identification of a new c.1024G>A *SIL1* gene variant. Sanger sequencing electropherograms showing the *SIL1* gene region containing the c.1024G>A variant. The variant base in the proband and her parents is highlighted. UNPUBLISHED DATA

3.3.1 *In Silico* Prediction and Experimental Evidence of Structural Impact for The c.1024G>A Variant

To understand the potential consequences of the identified c.1024G>A variant, we employed *in silico* algorithms to predict its impact on protein structure and function. Polymorphism Phenotyping v2 [42] (<http://genetics.bwh.harvard.edu/pph2/>) predicted the variant to be “probably damaging” with a score of 1 (on a 0-to-1 scale). Similarly, Mutation Taster analysis (<https://www.mutationtaster.org/>) indicated a very high probability (0.99) that this variant is disease-causing.

The c.1024G>A variant in the *SIL1* gene results in the substitution of a highly conserved glutamic acid (Glu) with a lysine (Lys) at position 342 of the protein (p.E342K). This amino acid change, replacing a negatively charged residue with a positively charged one within the helical domains of the armadillo repeats, could potentially disrupt protein structure and function. Thus, thanks to Luca Federici (University “G. d’Annunzio of Chieti-Pescara) we generated a structural model of human Sil1 using AlphaFold [43], which revealed the position of p.E342K approximately in the centre of a helix located in the middle of the concave surface implicated in BiP recognition at C-terminal folded domain (Figure 15A).

A zoomed view around residue 342 showed the position and electrostatic interactions of the mutated amino acid (Figure 15B). In the wild-type (WT) protein, Glu342 sits within a positively charged pocket created by His299 and Arg298, and forms hydrogen bonds with His299 and Gln304. The replacement of Glu with Lys at position 342 introduces multiple electrostatic repulsions, leading to localised structural instability. This effect was confirmed by the web server "Impact of Non-Synonymous Variations on Protein Stability-MultiDimension" (INPS-MD; <http://inpsmd.biocomp.unibo.it>) [44], which measured a change in protein stability ($\Delta\Delta G$) of -1.062 kcal/mol.

Figure 15C presents a structural superposition of our generated human Sil1 model (orange) with the complex between yeast Sil1 (cyan) and BiP (green) (10.2210/pdb3QML/pdb [3]). Although Glu342 doesn't directly bind BiP, it is part of a helix crucial for forming a BiP-binding cleft in Sil1. Therefore, it is reasonable to hypothesize that even a localized destabilization of this specific region in Sil1, such as that predicted for the p.E342K variant, could potentially impair or reduce the affinity for BiP.

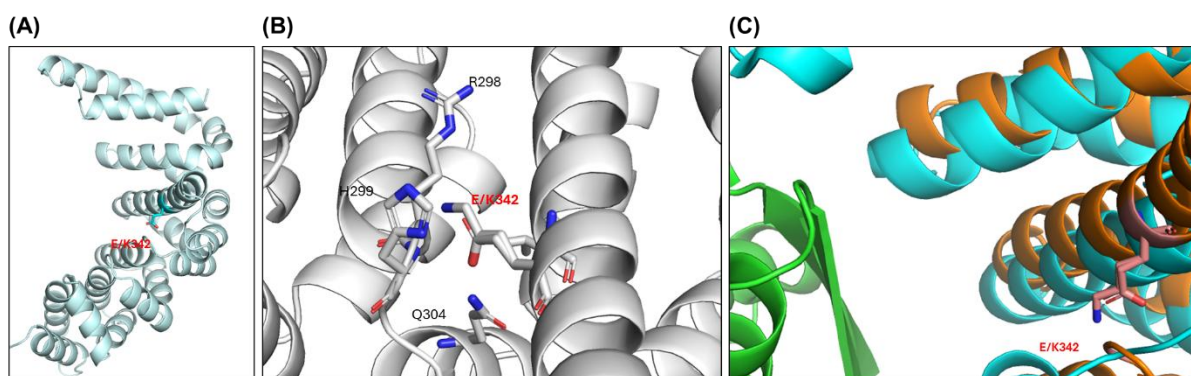


Figure 15 Three-dimensional analysis of the Sil1 p.E342K variant by AlphaFold modelling. A) AlphaFold modelling of the Sil1 p.E342K variant, with the position of the altered amino acid marked. B) Zoomed-in view of the region surrounding amino acid 342 of Sil1, illustrating the position and electrostatic interactions of the mutant residue. C) Superposition of the human Sil1 model (shown in orange) with the complex between yeast Sil1 (cyan) and BiP (green). UNPUBLISHED DATA

To gain a broader view of the 3D organisation of p.E342K Sil1 protein, we have enlisted the expertise of Adriana Erica Miele (Institute of Analytical Sciences, Lyon). In particular, Laura Amodei from our research group collaborated personally with her to measure the circular dichroism (CD) spectra of recombinant Sil1 WT and p.E342K proteins.

Surprisingly, these proteins, expressed in *E. coli* with a GST tag and purified by affinity chromatography, revealed significant differences: the p.E342K variant was less soluble and

stable than the WT. In addition, CD analysis showed that while WT had a mixed alpha-beta structure, p.E342K displayed a loss of alpha-helical content, resembling a coiled-coil fold, potentially explaining its instability (data not shown).

In summary, our integrated *in silico* and structural analyses, combined with biochemical evidence of instability and altered secondary structure, support the hypothesis that the novel p.E342K variant in *SIL1* is likely pathogenic.

3.3.2 Cellular Biomarkers Indicating The Pathogenicity of The P.E342K *SIL1* Variant

Considering that pathological *SIL1* variants exhibit abnormal migration in the native electrophoresis gel and form aggregates when overexpressed in COS7 and HEK293 cells [45], [46], [47], we decided to perform similar experiments to determine the p.E342K behaviour. Firstly, COS7 were transiently transfected to express the p.E342K variant, the WT (used as negative control), and the pathogenic p.L457P mutant of Sil1 (used as positive control), and Western blotting confirmed comparable expression levels of these proteins within the cells (Figure 16A). However, the evaluation of the conditioned medium from transfected COS7 revealed that both p.E342K and p.L457P variants were released into the culture medium in approximately 10-fold lower amounts than Sil1 WT, suggesting their compromised solubility (Figure 16B). In parallel, we monitored these proteins under native conditions, and Western blot analysis of native gel electrophoresis showed significantly reduced levels of both p.E342K and p.L457P compared to wild-type Sil1 (Figure 16C). To assess whether this phenomenon depended on the poor solubility of the mutant proteins and not on the different expressions, we treated the pellets remaining from native lysis procedure with a strongly denaturing urea buffer and analysed them through Western blotting. Results indicated a selective solubility of WT Sil1 over the variants (Figure 16D).

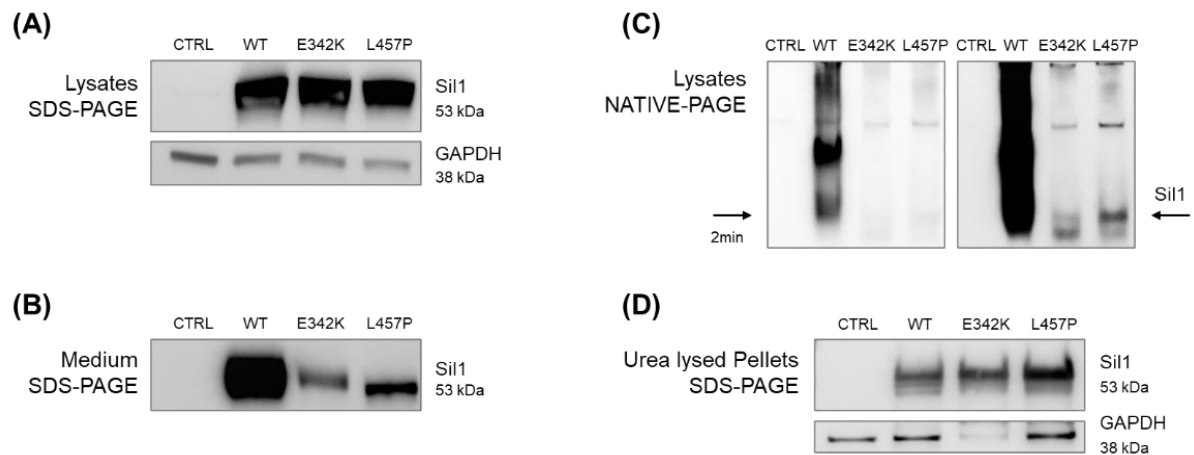


Figure 16 Solubility analysis of the Sil1 p.E342K variant. COS7 cells were transiently transfected with SIL1 WT, E342K, and L457P mutants. (A) Western blot analysis of whole-cell lysates from mock (CTRL) and WT, E342K, and L457P transfected cells showed comparable expression levels of all three constructs. GAPDH expression was analysed as loading control. (B) Western blotting of conditioned media revealed a reduced release of the E342K and L457P mutants into the culture medium. (C) COS7 cells transfected as described in A were lysed in native buffer and analysed by Native-PAGE and Western blotting. The left and right panels show the Western blotting CTRL, WT, E342K, and L457P developed (exposed) for 2 and 10 minutes, respectively. (D) Western blotting of urea-lysed pellet obtained from the native lysis analysed in C. GAPDH expression was analysed as loading control. UNPUBLISHED DATA

Finally, immunofluorescence microscopy showed a significant increase in intracellular Sil1 aggregates in COS7 expressing either p.E342K or p.L457P compared to those expressing WT protein (Figure 17). These experiments collectively demonstrated that p.E342K exhibits features more similar to the known pathological mutant p.L457P rather than the WT protein, strongly supporting its classification as a pathological form of Sil1.

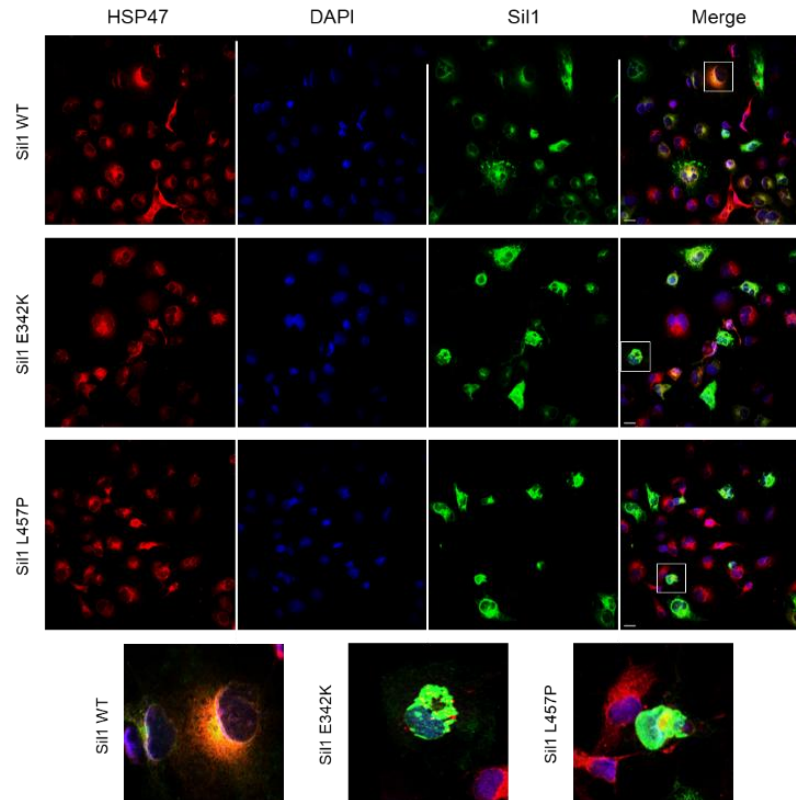


Figure 17 Confocal immunofluorescence analysis of COS7 cells transiently transfected with SIL1 WT, E342K, and L457P mutants. The cells were fixed and stained for Sil1 (green), HSP47 (red) as an ER marker, and DAPI (blue) to visualise the nuclei. Merged images are also shown. Enlarged images of the cells are shown in the lower part of the figure to better appreciate the different intracellular distribution of Sil1 WT and mutants. Scale bar: 20 μm. UNPUBLISHED DATA

3.3.3 Cellular Impact of The P.E342K SIL1 Variant in Patient Derived Fibroblasts

In order to determine the cellular effects of the p.E342K variant, we generated a primary culture of skin fibroblasts isolated from the Greek patient (HF-P2, human fibroblasts-patient 2), and compared them to healthy control (HF-CTRL) and to the other patient-derived fibroblast cell line, carrying the R111X mutation (HF-MSS, from now HF-P1).

Firstly, we examined their cell cycle, proliferation rate and motility, and we found out that HF-P2 cells showed a cell cycle distribution and proliferation rate comparable to controls, and also cell motility was unaffected. These findings were in contrast to HF-P1, which exhibited reduced growth rate and reduced motility in our previous studies (Figure 12C and D) [21, 41].

Western blot and immunofluorescence analyses revealed that p.E342K Sil1 levels were reduced by about 80% compared to WT protein, supporting the hypothesis of protein instability (Figure

18A and B). Notably, Sil1 protein was undetectable in HF-P1 cells, because of the nonsense mutation R111X. To determine whether this reduced protein expression was due to decreased gene transcription, we conducted reverse transcriptase quantitative PCR (RT-qPCR). Findings showed that Sil1 mRNA expression levels in HF-P2 were higher than control fibroblasts, while in HF-P1 cells mRNA expression was reduced 5-fold, probably because of the activation of nonsense-mediated mRNA decay (Figure 18C).

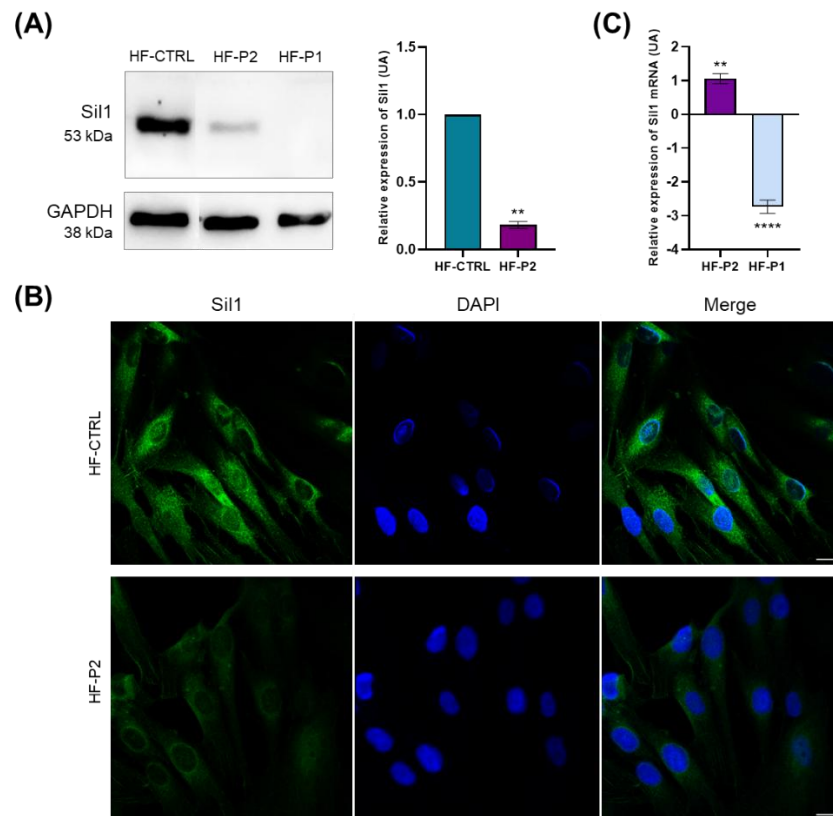


Figure 18 Sil expression analysis in HF-P2 primary skin fibroblast. (A) Western blotting of control (HF-CTRL), HF-P2 and HF-P1 primary skin fibroblasts. The quantification of Sil1 expression is shown in the graph on the right. GAPDH expression was analysed as loading control. Unpaired t-test was used to compare Sil1 expression of HF-P2 to HF-CTRL. *** $p < 0.001$. (B) Confocal immunofluorescence analysis of Sil1 in HF-P2 cells. The cells were fixed and stained for Sil1 (green), and DAPI (blue) to visualize the nuclei. Merged images are also shown. Scale bar: 20 μ m. (C) Quantitative RT-PCR analysis of Sil1 mRNA expression levels in HF-P1 and HF-P2. Results are expressed as the log of fold changes relative to control fibroblasts, calculated using the $\Delta\Delta Ct$ method, with GAPDH as the reference gene. Unpaired t-test was used to compare ΔCt of SIL1 gene of both HF-P2 and HF-P1 with HF-CTRL. ** $p < 0.01$, **** $p < 0.0001$. UNPUBLISHED DATA

We hypothesised that reduced Sil1 protein in HF-P2 fibroblasts resulted from post-translational degradation, thus we decided to treat these cells with the proteasomal inhibitor MG-132. This treatment, indeed resulted in a significant increase in Sil1 protein expression in Greek patient cells, while it didn't affect controls, as revealed by Western blotting (Figure 19A and B) and

immunofluorescence microscopy (Figure 19C). In addition, IF analysis showed the co-localisation of Sil1 with the lysosomal marker LAMP2 in both MG132-treated HF-P2 and HF-CTRL, suggesting also lysosomal degradation for unfolded Sil1 (Figure 19C).

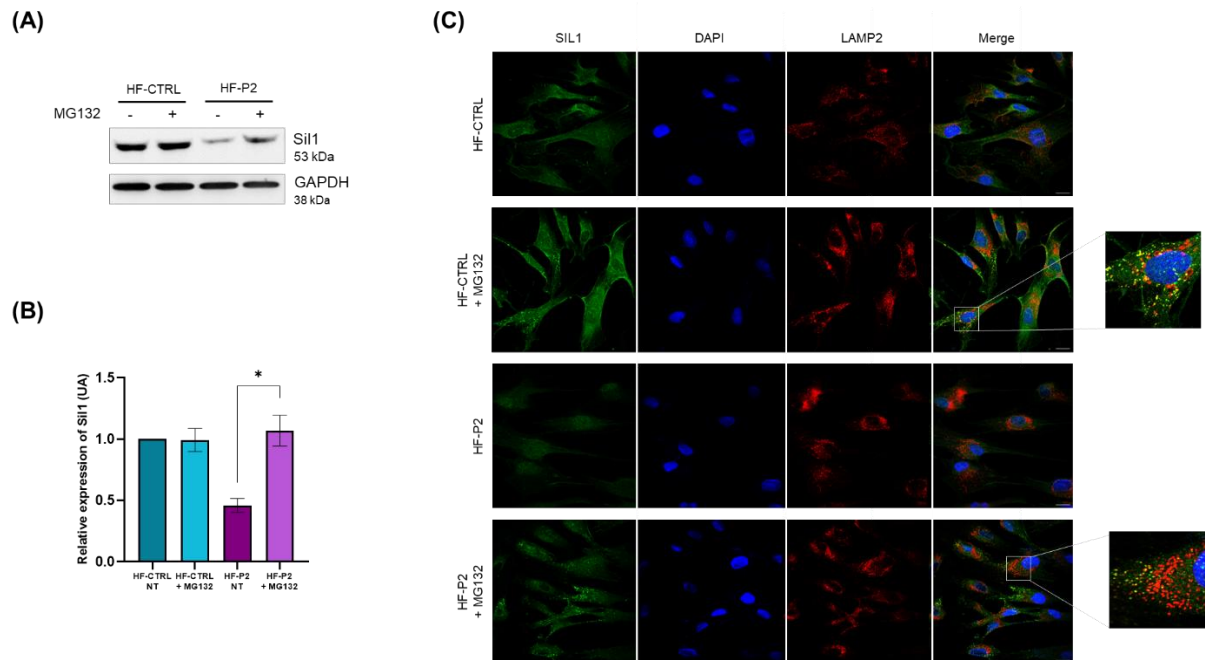


Figure 19 MG132 treatment. (A) Controls and HF-P2 were treated with 20 μ M of MG-132 20 μ M, for 6 hours, lysed and processed for Western blotting. GAPDH expression was analysed as loading control. (B) Quantification of Sil1 expression (from 3 experiments). To evaluate the different expression of Sil1 in HF-P2 due to MG132 treatment, unpaired t-test was performed. * $p < 0.05$. (C) Controls and HF-P2 cells were treated as in A. The cells were fixed and stained for Sil1 (green), LAMP2 as lysosomal marker (red) and DAPI (blue) to visualise the nuclei. Merged images are also shown. Scale bar: 20 μ m. UNPUBLISHED DATA

Furthermore, we assessed the expression of 12 genes known to be altered in HF-P1 (Figure 9C) [41]. Notably, all these genes were also dysregulated in HF-P2, with 10 of them showing the same trend (Figure 20). However, the extent of these changes was less pronounced in HF-P2 than in HF-P1, suggesting that p.E342K might be a milder pathological variant than R111X.

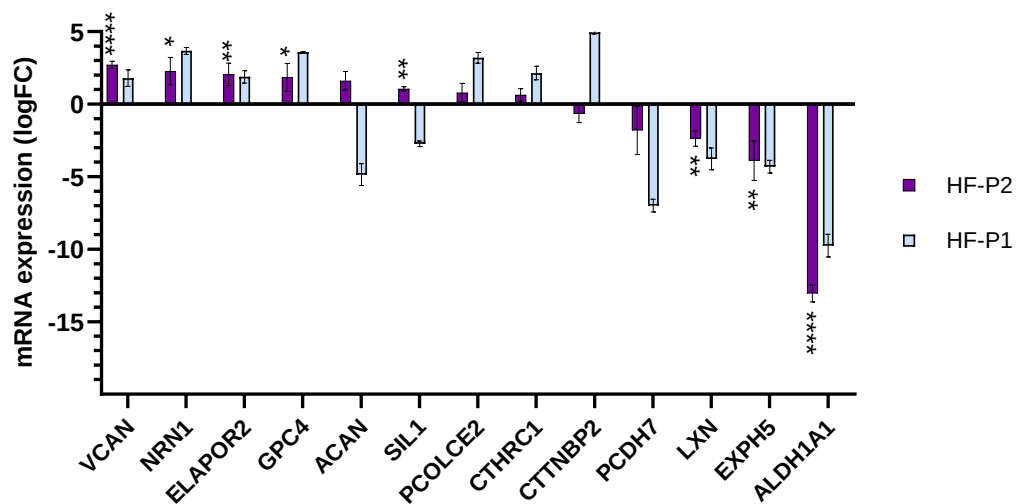


Figure 20 RT-qPCR analysis of selected genes. Total RNA was extracted from controls, HF-P1 and HF-P2 cells and gene expression was analysed by qPCR. Data are expressed as the log of fold change relative to control, calculated using the $\Delta\Delta C_t$ method, with GAPDH as the reference gene. Multiple unpaired t-test was used to compare ΔC_t of each gene between HF-P2 and HF-CTRL * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. UNPUBLISHED DATA

Finally, to determine if the p.E342K variant induced morphological changes in patient fibroblasts, we proceeded to ultrastructural examination of the Greek patient fibroblasts, helped by Laura Pietrangelo. TEM analysis of HF-P2 cells revealed an abundant presence of variously sized peripheral vesicles, mainly empty in contrast to HF-P1 (Figure 21, central panel), while in control cells these structures were absent. Moreover, the cytoplasm of HF-P2 showed depots of lipid droplets (asterisks in Figure 21) and was characterized by clearer areas (white arrow in upper panels of Figure 21), outlined by ER or mitochondria, that at higher magnification appeared to be cytoplasmic deposits of proteinaceous material (letter f in lower panels of Figure 21). TEM re-examination of HF-P1 confirmed the presence of lipid droplets and fibrotic material in both patient lines (right panels of Figure 21), thus revealing novel cellular alterations associated with MSS and providing further support for the pathogenicity of p.E342K variant.

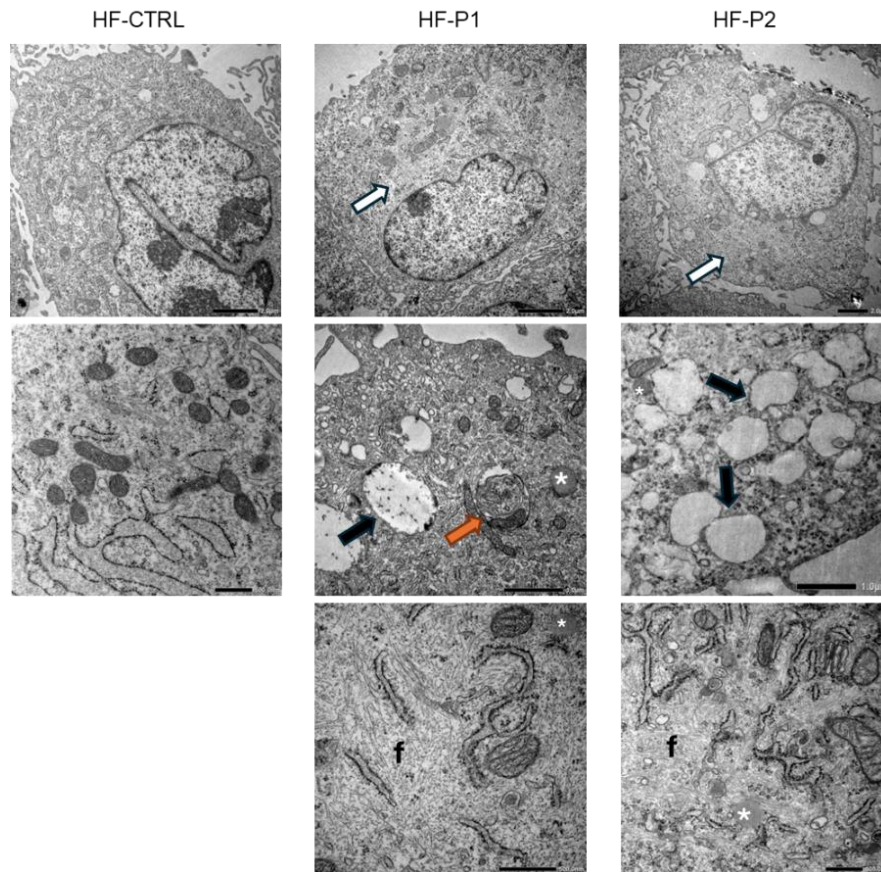


Figure 21 Ultrastructural analysis of HF-P2 fibroblast. Transmission electron microscopy (TEM) of control, HF-P1, and HF-P2 cells. HF-P1 and HF-P2 cells exhibited cytoplasmic abnormalities, including clear cytoplasmic material accumulation (white arrows), multilamellar bodies (orange arrows), empty vacuoles (black arrows), lipid droplets (*), and aberrant fibrillar material (f). Scale bars: 2 μm , 1 μm , 500 nm. UNPUBLISHED DATA

3.3.4 Proteomic Evidence of Altered Proteostasis Characteristic of Marinesco-Sjögren Syndrome in P.E342K Fibroblasts

In order to assess the protein signature of HF-P2, we performed a proteomic analysis in collaboration with our mass spectrometry facility (Piero Del Boccio and colleagues).

We identified about 2240 proteins, with 122 proteins downregulated and 107 upregulated, for a total of 229 DE proteins compared to control fibroblasts ($p < 0.05$), related to cytoskeleton, extracellular matrix and metabolite interconversion enzymes.

Figure 22 shows respectively the heat map (A), the volcano plot (B), and the top-modulated proteins ranked by their fold-change (C).

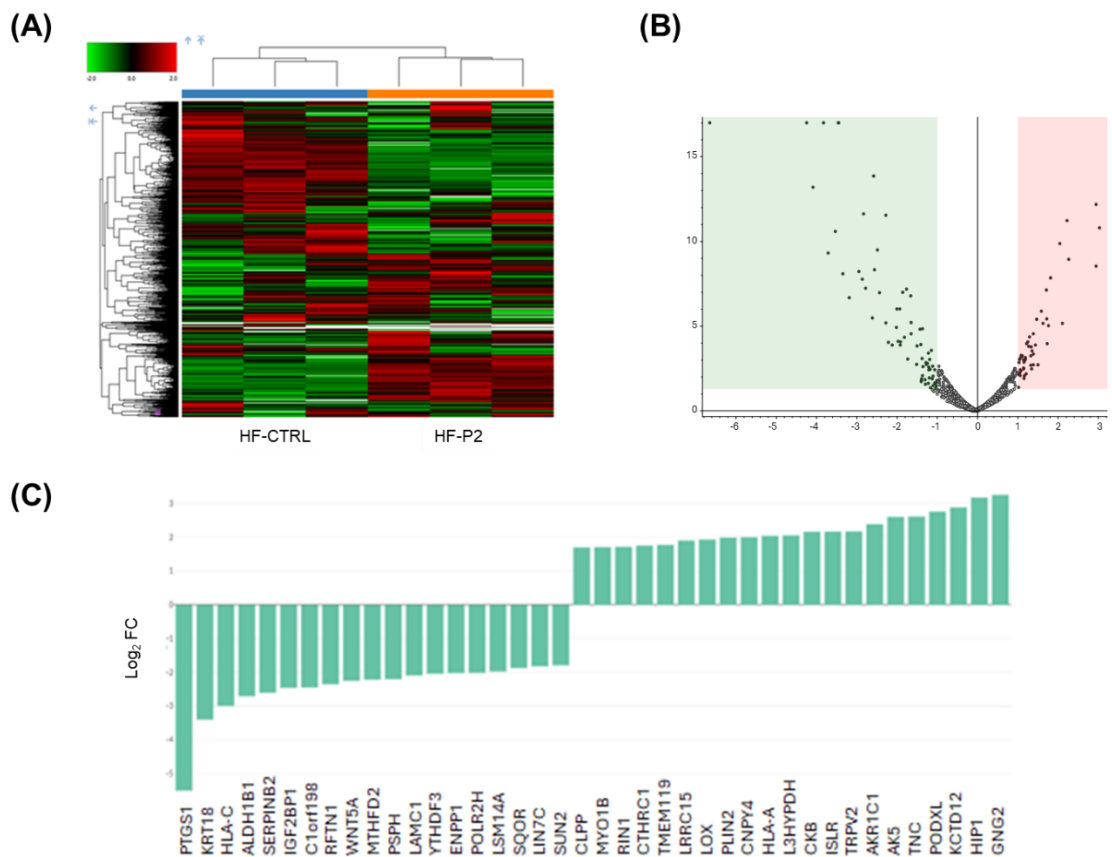


Figure 22 Comparative proteomic analysis of controls and HF-P2 cells. A-B) Heat map and volcano plot of differentially expressed proteins in triplicate experiments. Expression ratios and p-values are presented on a logarithmic scale in the volcano plot. C) The top 10 upregulated and downregulated DE proteins are expressed as log2 fold changes. UNPUBLISHED DATA

We performed a comparison between the DE proteins in HF-P1 and the DE proteins generated by a new proteomic analysis of HF-P1, which revealed a general correlation ($r=0.5477$) in the observed alterations (Figure 23A). Particularly, the most similar altered proteins are displayed in Figure 23B, and this strong correlation is an additional of the pathogenicity of the p.E342K variant.

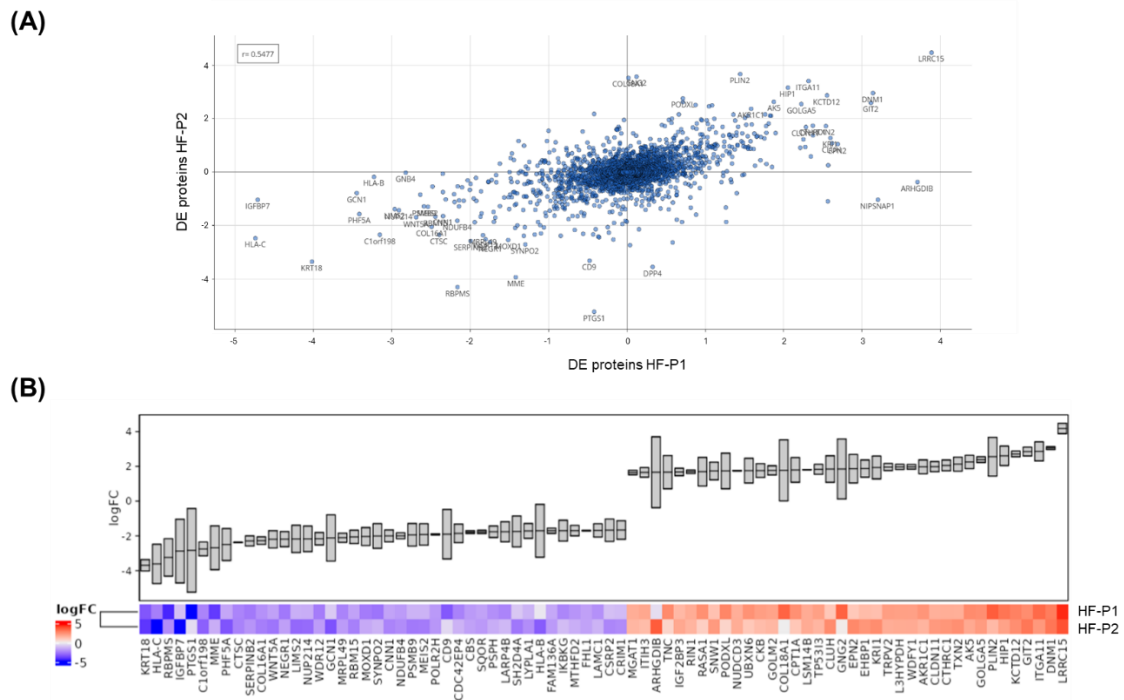


Figure 23. Comparative analysis of DE proteins identified in HF-P1 and HF-P2 cells. A) Correlation analysis of the differentially expressed (DE) proteins identified by proteomics in HF-P1 and HF-P2 cells. B) Comparison of the fold changes (on a logarithmic scale) in the 40 most upregulated and 40 most downregulated proteins identified by proteomics in HF-P1 and HF-P2 cells. UNPUBLISHED DATA

To further validate our proteomics data, we confirmed the altered expression of some key proteins through Western blotting: PDZ and LIM domain 5 (PDLIM5), potassium channel tetramerization domain containing 12 (KCTD12), LIM domain 1 and a half (FHL1), collagen triple helix repeat containing 1 (CTHRC1) and creatine kinase B-type (CKB) (Figure 24).

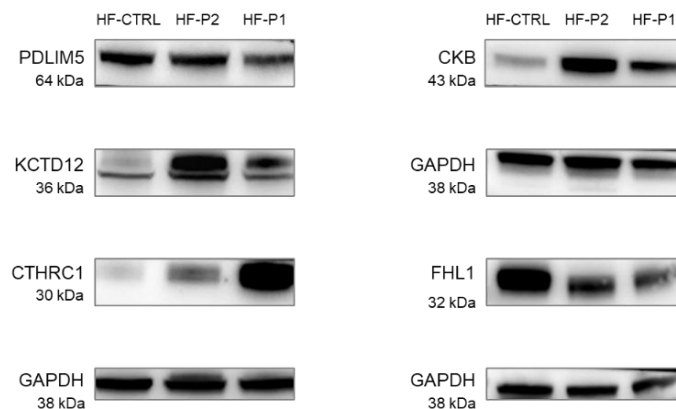


Figure 24 Validation of selected DE proteins by Western blot. Control (HF-CTRL) and HF-P2 cells were subjected to Western blot analysis, to analyse the expression of PDLIM5, KCTD12, CTHRC1, CKB and FHL1 as indicated. GAPDH expression was analysed as loading control. UNPUBLISHED DATA

To better understand the functional relationships among these proteins, we consulted the STRING database, which revealed a significant PPI interaction network (Figure 25) and a significant PPI enrichment (p -value: $< 1.0e-16$).

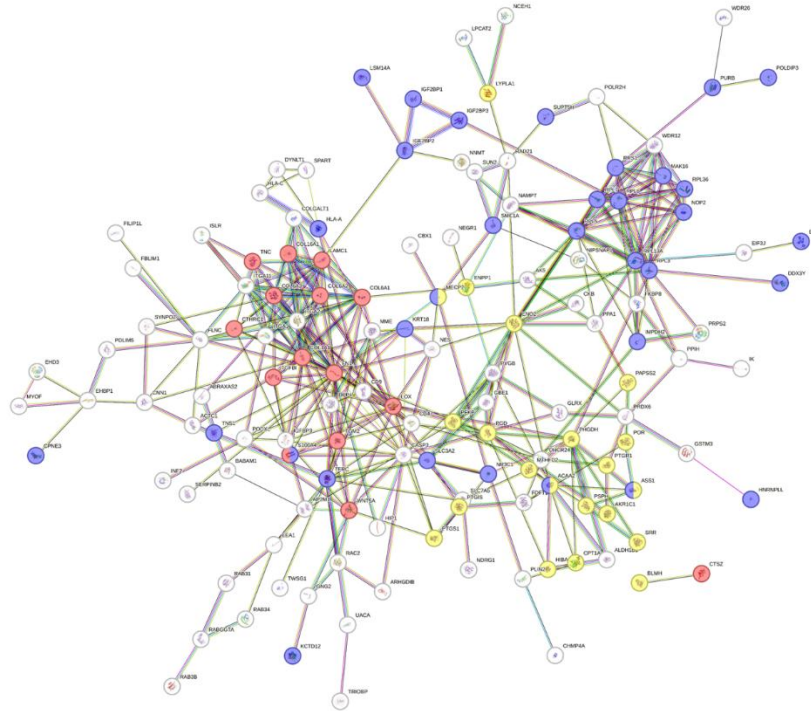


Figure 25 Protein-protein interaction network of DE proteins identified in HF-P2 cells. Proteins associated with the biological process 'Oxoacid metabolic process,' the molecular function 'RNA binding,' and the cellular component 'Collagen-containing extracellular matrix' are marked in yellow, blue, and red, respectively. UNPUBLISHED DATA

GO enrichment highlighted potentially affected biological processes in HF-P2, such as ribosomal large subunit biogenesis, cytoplasmic translation, regulation of cell adhesion, and oxoacid metabolic process (data not shown). Notably, a large proportion of these processes overlapped with those enriched in our previous proteomics (92%) and transcriptomics (53%) of HF-P1 [21, 41], as well as in the proteomics (66%) of the *Sil1^{Gt}* mouse quadriceps [25]. Molecular function enrichment analysis indicated potential involvement of extracellular matrix structural constituents, collagen binding, mRNA binding, and cell adhesion molecule binding, while enriched cellular components included the cytosolic large ribosomal subunit, protein complexes involved in cell adhesion, collagen trimer, phagocytic vesicle membrane, focal adhesion, endocytic vesicle membrane, and collagen-containing extracellular matrix (data not shown).

Furthermore, enrichment of Reactome and KEGG molecular pathways confirmed some

alterations consistent with previous MSS findings [21, 25] and the above Gene Ontologies (data not shown).

In conclusion, our comprehensive proteomic analysis provides strong evidence that the p.E342K variant induces a pathological cellular phenotype that closely resembles the proteomic signature previously reported for MSS. Thus, based on the collective findings, we determined that the new p.E342K variant is pathological and responsible for MSS, and appears to have a less pronounced cellular effect than the p.R111X nonsense variant.

3.4 Assessment of The Woozy Mouse Model Phenotype

UNPUBLISHED DATA

Despite the knowledge generated by previous studies on the woozy mouse model [14, 24, 48, 49], several features remain unexplored, and the extent to which it fully reflects the spectrum of MSS clinical characteristics is still not entirely clear.

Particularly, the timeline of cellular and molecular changes across different brain regions remains poorly defined, with little exploration of stress-related responses beyond the cerebellum. Secondly, the interaction between protein aggregation, cellular stress, and neuronal dysfunction requires further clarification, particularly in identifying critical intervention windows to prevent disease onset rather than merely slowing progression. In addition, this model may not fully reflect the variability and age-dependent progression of symptoms seen in human patients.

In order to have a more comprehensive understanding of the impact of the *SIL1* mutations on this animal model, we undertook an initial explorative investigation of the motor and cognitive functions of our *Sil1^{wz}* mice compared to controls (*Sil1* heterozygous mice, *Sil1^{ht}*) across a broad range, from 5 to 26 weeks, while also examining the specific influence of sex. Moreover, we analysed the molecular profiles in the quadriceps and soleus muscles of these animals at the 26th week.

In particular, I primarily contributed to this work by carrying out the motor and behavioural experiments on animal models.

3.4.1 Assessment of Motor Coordination and Strength in The *Sil1^{wz}* Mouse Model

To evaluate sensorimotor deficits, we first performed the accelerated rotarod test on *wz* mice from the 5th to 14th week of age. Starting from the 9th week, they exhibited a significantly shorter latency time compared to the control group, which continued to decrease until week 14 (Figure 26A). When analysing sexes separately, male *Sil1^{wz}* mice showed a significant decrease in latency time compared to male *Sil1^{ht}* mice starting from the 8th week (Figure 26B), while in females the significant difference between *Sil1^{wz}* and *Sil1^{ht}* was observed from the 12th week onwards (Figure 26C).

Comparing motor performance between sexes within each genotype revealed that females generally performed better than males in both *Sil1^{ht}* and *Sil1^{wz}* groups (Figure 26D and E). However, this difference only reached statistical significance for *Sil1^{wz}* females compared to

Sil1^{wz} males at the 11th week (Figure 26E). Weekly average times and specific *p*-values for each week are detailed in Table 7. Our results were in line with previous published results [24, 50]. The reduced latency to fall on the rotarod could suggest a reduction in physical resistance, but this test primarily assesses locomotor coordination and balance, and not muscle strength [51]. Therefore, we decided to further investigate motor functions through other specific tests.

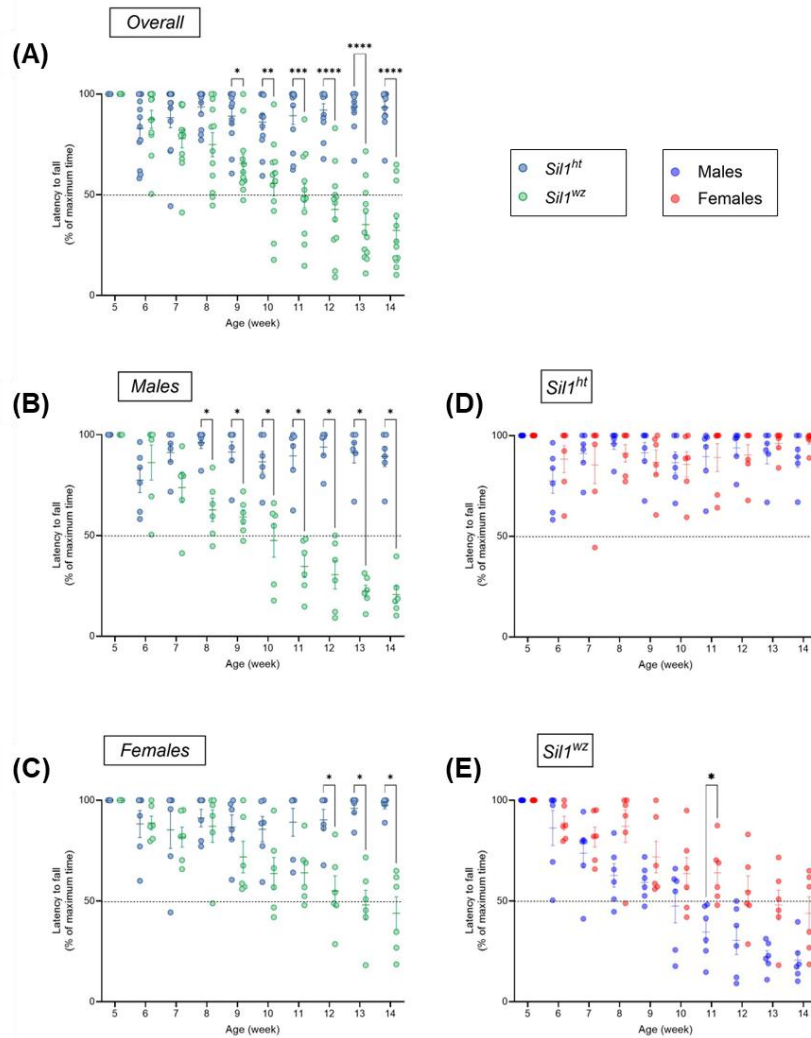


Figure 26 Accelerating rotarod test results – Latency to fall in the overall population (A), male (B), female (C), *Sil1^{ht}* (D), and *Sil1^{wz}* mice (E). Data are represented as scattered dot plots (mean $\pm$ SEM, of each group), and expressed as the percentage of the maximum time for the individual animal. *Sil1^{wz}* = green circles; *Sil1^{ht}* = blue circles; Males = blue circles; Females = red circles. Significant differences are indicated (**p*<0.05, ***p*<0.01, ****p*<0.005, *****p*<0.001). UNPUBLISHED DATA

	Mean (% $\pm$ SEM)	Mean (% $\pm$ SEM)	p value
<i>Overall</i>			
Week	<i>Sil1^{ht}</i>	<i>Sil1^{wz}</i>	
5	100 $\pm$ 0.00	100 $\pm$ 0.00	>0.9999
6	82.88 $\pm$ 4.64	87.49 $\pm$ 4.46	0.7206
7	88.29 $\pm$ 4.98	77.77 $\pm$ 4.40	0.0815
8	93.61 $\pm$ 2.58	74.95 $\pm$ 5.99	0.0815

9	89.08 ± 3.88	65.55 ± 4.54	0.0112
10	86.11 ± 3.91	55.59 ± 6.02	0.0030
11	89.29 ± 4.37	49.35 ± 5.86	0.0004
12	92.09 ± 3.16	42.73 ± 6.14	< 0.0001
13	93.61 ± 2.83	35.23 ± 5.41	< 0.0001
14	93.42 ± 2.83	32.37 ± 5.59	< 0.0001

Males

Week	<i>Sil1^{ht}</i>	<i>Sil1^{wz}</i>	
5	100 ± 0.00	100 ± 0.00	>0.9999
6	77.51 ± 6.17	86.24 ± 8.68	0.4129
7	91.19 ± 4.39	73.78 ± 7.37	0.1767
8	96.04 ± 2.87	62.74 ± 5.79	0.0215
9	91.45 ± 5.21	59.23 ± 3.61	0.0215
10	86.56 ± 5.28	47.53 ± 8.35	0.0214
11	89.48 ± 6.01	34.67 ± 5.46	0.0214
12	93.83 ± 3.97	30.50 ± 7.02	0.0214
13	91.11 ± 5.09	22.43 ± 2.98	0.0214
14	89.27 ± 5.01	20.72 ± 4.25	0.0214

Females

Week	<i>Sil1^{ht}</i>	<i>Sil1^{wz}</i>	
5	100 ± 0.00	100 ± 0.00	>0.9999
6	88.26 ± 6.7	88.75 ± 3.39	0.9745
7	85.39 ± 9.31	81.76 ± 4.96	0.6553
8	91.19 ± 4.32	87.16 ± 8.04	0.9958
9	86.72 ± 6.07	71.87 ± 7.85	0.6524
10	85.66 ± 6.26	63.66 ± 7.99	0.3316
11	89.10 ± 6.92	64.03 ± 5.93	0.1552
12	90.34 ± 5.20	54.96 ± 7.55	0.0341
13	96.10 ± 5.60	48.04 ± 7.39	0.0214
14	97.57 ± 1.76	44.02 ± 8.08	0.0214

Sil1^{ht}

Week	Males	Females	
5	100 ± 0.00	100 ± 0.00	>0.9999
6	77.51 ± 6.17	88.26 ± 6.7	0.8583
7	91.19 ± 4.39	85.39 ± 9.31	0.9988
8	96.04 ± 2.87	91.19 ± 4.32	0.9982
9	91.45 ± 5.21	86.72 ± 6.07	0.9847
10	86.56 ± 5.28	85.66 ± 6.26	0.9988
11	89.48 ± 6.01	89.10 ± 6.92	0.9847
12	93.83 ± 3.97	90.34 ± 5.20	0.9988
13	91.11 ± 5.09	96.10 ± 5.60	0.9847
14	89.27 ± 5.01	97.57 ± 1.76	0.9807

Sil1^{wz}

Week	Males	Females	
5	100 ± 0.00	100 ± 0.00	>0.9999
6	86.24 ± 8.68	88.75 ± 3.39	0.8448
7	73.78 ± 7.37	81.76 ± 4.96	0.8431
8	62.74 ± 5.79	87.16 ± 8.04	0.3007
9	59.23 ± 3.61	71.87 ± 7.85	0.8431
10	47.53 ± 8.35	63.66 ± 7.99	0.8431
11	34.67 ± 5.46	64.03 ± 5.93	0.0425
12	30.50 ± 7.02	54.96 ± 7.55	0.3007
13	22.43 ± 2.98	48.04 ± 7.39	0.3007
14	20.72 ± 4.25	44.02 ± 8.08	0.3007

Table 7 Accelerating Rotarod test results observed between the 5th and the 14th week. Data are reported as group mean (s) ± SEM, obtained by three trials of each animal and represented as the percentage of the individual maximum latency time. Significant results are highlighted in bold (Mann-Whitney test with Holm-Sidak correction for multiple comparison). UNPUBLISHED DATA

Secondly, we performed the beam walking test, which is considered the most sensitive for

monitoring disease development [52, 53]. From the 7th week onwards, *Sil1^{wz}* showed a significantly increased time to traverse the beam compared to control mice *Sil1^{ht}*. This difference progressively increased until the end of the test (Figure 27A). Data analysis by sex revealed that male *Sil1^{wz}* exhibited a significantly longer beam traversal time compared to male *Sil1^{ht}* mice from the beginning of the test at the 5th week (Figure 27B). In contrast, female *Sil1^{wz}* mice started showing significantly longer traversal times compared to their *Sil1^{ht}* counterparts from the 9th week (Figure 27C). This delay seems to be due to a high variability of *Sil1^{ht}* females who may not perform “as well” as *Sil1^{ht}* males during the first weeks of the test. Similarly, the number of contralateral foot slips during beam traversal was significantly higher in the overall *Sil1^{wz}* population from the first week of testing. This increase was significant in males from the second week (Figure 27E) and from the beginning of the test in females (Figure 27F).

No significant differences were observed when comparing motor performance between males and females within the same genotype, thus demonstrating the high specificity of this test in measuring muscle coordination independently from sex contribution (Figure 27G-J). The weekly average time to traverse the beam, the number of foot slips, and the corresponding p-values for each week are detailed in Table 8.

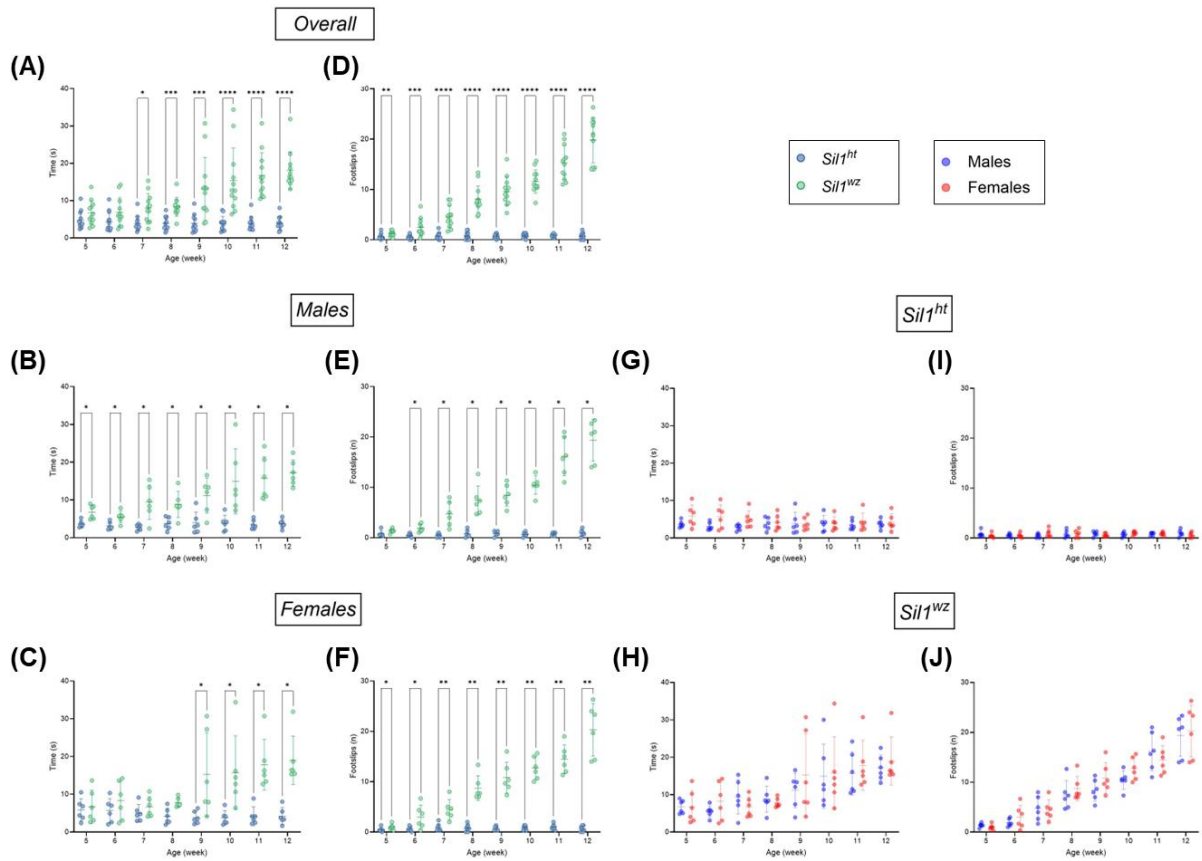


Figure 27 Beam walking test results – Time to traverse the bar and number of contralateral falls in the overall population (A,D), male (B,E), female (C,F), Sil1^{ht} (G,I), and Sil1^{wz} mice (H,J). Data are represented as scattered dot plots (mean $\pm$ SEM, of each group). Sil1^{wz} = green circles; Sil1^{ht} = blue circles; Males = blue circles; Females = red circles. Significant differences are indicated (* p <0.05, ** p <0.01, *** p <0.005, **** p <0.001). UNPUBLISHED DATA

	Mean (s ± SEM)		p value	Mean (n ± SEM)		p value
Overall						
	Time			Misstep		
Week	Sil1 ^{ht}	Sil1 ^{wz}		Sil1 ^{ht}	Sil1 ^{wz}	
5	4.76 ± 0.69	6.74 ± 0.93	0.1158	0.62 ± 0.16	1.18 ± 0.15	0.0089
6	4.40 ± 0.76	6.90 ± 1.13	0.1158	0.50 ± 0.11	2.45 ± 0.51	0.0002
7	3.88 ± 0.58	8.03 ± 1.11	0.0135	0.69 ± 0.19	4.58 ± 0.62	< 0.0001
8	3.94 ± 0.52	8.33 ± 0.73	0.0005	0.81 ± 0.21	8.06 ± 0.77	< 0.0001
9	3.87 ± 0.65	13.18 ± 2.42	0.0006	0.69 ± 0.13	9.64 ± 0.82	< 0.0001
10	3.81 ± 0.55	15.36 ± 2.53	< 0.0001	0.81 ± 0.13	11.58 ± 0.67	< 0.0001
11	3.82 ± 0.55	16.75 ± 1.75	< 0.0001	0.81 ± 0.10	15.31 ± 0.97	< 0.0001
12	3.97 ± 0.50	18.08 ± 1.45	< 0.0001	0.75 ± 0.18	19.81 ± 1.30	< 0.0001
Males						
	Time			Misstep		
Week	Sil1 ^{ht}	Sil1 ^{wz}		Sil1 ^{ht}	Sil1 ^{wz}	
5	3.65 ± 0.37	6.78 ± 0.75	0.0426	0.81 ± 0.25	1.36 ± 0.17	0.1061
6	3.10 ± 0.42	5.52 ± 0.63	0.0426	0.50 ± 0.14	1.89 ± 0.32	0.0172
7	2.77 ± 0.32	9.40 ± 1.87	0.0448	0.45 ± 0.17	4.72 ± 1.00	0.0172
8	3.73 ± 0.67	8.83 ± 1.41	0.0448	0.78 ± 0.31	7.39 ± 1.18	0.0172
9	3.91 ± 1.18	11.14 ± 1.89	0.0448	0.83 ± 0.21	8.44 ± 0.89	0.0172
10	3.84 ± 0.87	14.93 ± 3.51	0.0257	0.67 ± 0.19	10.44 ± 0.74	0.0172
11	3.47 ± 0.51	15.67 ± 2.35	0.0172	0.78 ± 0.14	16.17 ± 1.58	0.0172
12	3.7 ± 0.50	17.17 ± 1.42	0.0172	0.95 ± 0.28	19.33 ± 1.69	0.0172
Females						

Time			Misstep		
Week	<i>Sil1^{ht}</i>	<i>Sil1^{wz}</i>		<i>Sil1^{ht}</i>	<i>Sil1^{wz}</i>
5	5.88 ± 1.21	6.70 ± 1.80	> 0.9999	0.43 ± 0.16	1.00 ± 0.23
6	5.71 ± 1.29	8.28 ± 2.10	0.7774	0.57 ± 0.17	3.00 ± 0.96
7	5.00 ± 0.93	6.67 ± 1.09	0.7774	0.85 ± 0.29	4.45 ± 0.83
8	4.15 ± 0.86	7.83 ± 0.50	0.0999	0.81 ± 0.27	8.72 ± 1.01
9	3.83 ± 0.69	15.22 ± 4.53	0.0426	0.66 ± 0.16	10.83 ± 1.26
10	3.79 ± 0.77	15.79 ± 3.97	0.0257	0.86 ± 0.16	12.72 ± 0.93
11	4.18 ± 1.00	17.83 ± 2.73	0.0172	1.00 ± 0.21	14.44 ± 1.16
12	4.23 ± 0.91	18.99 ± 2.62	0.0172	0.67 ± 0.22	20.28 ± 2.12

Time			Misstep		
Week	Males	Females		Males	Females
5	3.65 ± 0.37	5.88 ± 1.21	0.7500	0.81 ± 0.25	0.43 ± 0.16
6	3.10 ± 0.42	5.71 ± 1.29	0.8077	0.50 ± 0.14	0.57 ± 0.17
7	2.77 ± 0.32	5.00 ± 0.93	0.4156	0.45 ± 0.17	0.85 ± 0.29
8	3.73 ± 0.67	4.15 ± 0.86	0.9989	0.78 ± 0.31	0.81 ± 0.27
9	3.91 ± 1.18	3.83 ± 0.69	0.9975	0.83 ± 0.21	0.66 ± 0.16
10	3.84 ± 0.87	3.79 ± 0.77	>0.9999	0.67 ± 0.19	0.86 ± 0.16
11	3.47 ± 0.51	4.18 ± 1.00	0.9990	0.78 ± 0.14	1.00 ± 0.21
12	3.7 ± 0.50	4.23 ± 0.91	>0.9999	0.95 ± 0.28	0.67 ± 0.22

Time			Misstep		
Week	Males	Females		Males	Females
5	6.78 ± 0.75	6.70 ± 1.80	0.9918	1.36 ± 0.17	1.00 ± 0.23
6	5.52 ± 0.63	8.28 ± 2.10	0.9818	1.89 ± 0.32	3.00 ± 0.96
7	9.40 ± 1.87	6.67 ± 1.09	0.9818	4.72 ± 1.00	4.45 ± 0.83
8	8.83 ± 1.41	7.83 ± 0.50	0.9818	7.39 ± 1.18	8.72 ± 1.01
9	11.14 ± 1.89	15.22 ± 4.53	0.9940	8.44 ± 0.89	10.83 ± 1.26
10	14.93 ± 3.51	15.79 ± 3.97	0.9940	10.44 ± 0.74	12.72 ± 0.93
11	15.67 ± 2.35	17.83 ± 2.73	0.9818	16.17 ± 1.58	14.44 ± 1.16
12	17.17 ± 1.42	18.99 ± 2.62	0.9940	19.33 ± 1.69	20.28 ± 2.12

Table 8 Beam Walking test results observed between the 5th and the 12th weeks. Data are reported as group mean ± SEM, obtained by three trials of each animal. Significant results are highlighted in bold (Mann-Whitney test with Holm-Sidak correction for multiple comparison). UNPUBLISHED DATA

The third motor test we used to characterise this model is the pole test, which is a powerful tool to evaluate cerebellar dysfunctions. Specifically, apart from the total time taken to descend the pole, the time required for the mice to turn their head from up to down could be used as a specific indicator of cerebellar ataxia and motor coordination. As expected, woozy mice exhibited a significantly increased turning time (T-turn) once attached to the pole compared to controls at both the 14th and 16th week (Figure 28A). Similarly, the total time to descend the pole (T-total), measured after the mouse had fully turned downwards, was significantly increased in the *Sil1^{wz}* group at both the 14th and 16th week (Figure 28A).

In contrast to the rotarod and beam walking tests, the pole test revealed a clear sex-based difference. Specifically, males mirrored the overall group differences, showing significantly increased T-turn and T-total times in *Sil1^{wz}* compared to *Sil1^{ht}* at both time points (Figure 28B). One male from the *Sil1^{ht}* group was excluded due to consistent test failure. However, no statistically significant differences were observed between *Sil1^{wz}* and *Sil1^{ht}* females for either parameter at the considered time points (Figure 28C).

When comparing sexes within the same genotype, we didn't find significant differences in *Sil1^{ht}* animals (Figure 28D), whereas *Sil1^{wz}* females demonstrated significantly better performance than *Sil1^{wz}* males, exhibiting shorter T-turn times at both weeks and a shorter T-total time at the 14th week (Figure 28E). All group means and *p*-values are detailed in Table 9.

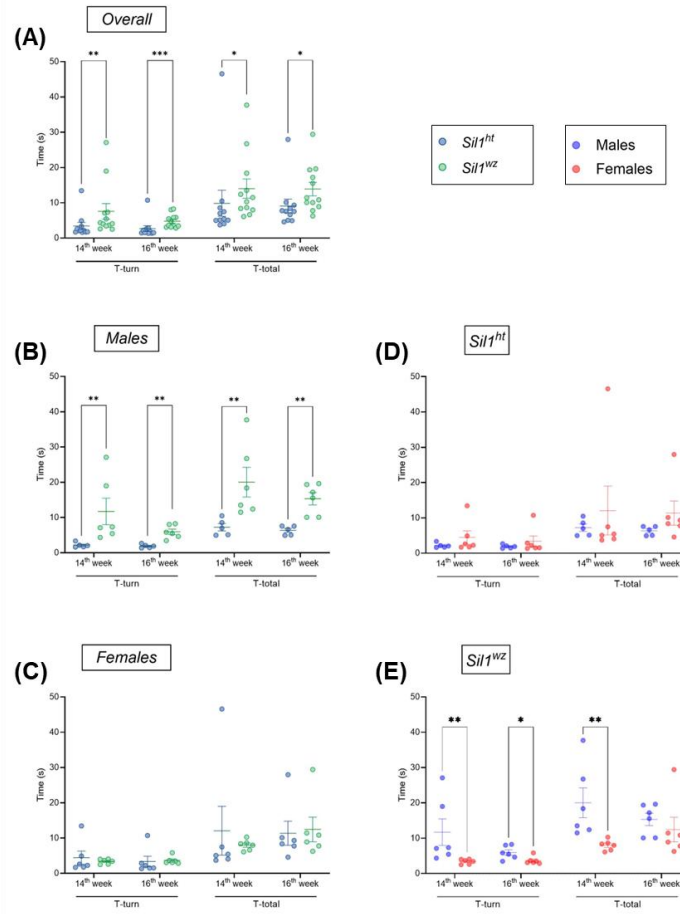


Figure 28 Pole test results – Turning (*T-turn*) and total (*T-total*) time at the 14th and 16th week of life in the overall population (a), male (b), female (c), *Sil1^{ht}* (d), and *Sil1^{wz}* mice (e). Data are represented as scattered dot plots (mean $\pm$ SEM, of each group). *Sil1^{wz}* = green circles; *Sil1^{ht}* = blue circles; Males = blue circles; Females = red circles. Significant differences are indicated (**p*<0.05, ***p*<0.01, ****p*<0.005). UNPUBLISHED DATA

	Mean (s ± SEM)		p value	Mean (s ± SEM)		p value
Overall						
	T-turn			T-total		
Week	Sil1 ^{ht}	Sil1 ^{wz}		Sil1 ^{ht}	Sil1 ^{wz}	
14	3.42 ± 1.05	7.55 ± 2.19	0.0090	9.87 ± 3.72	14.02 ± 2.72	0.0129
16	2.70 ± 0.82	4.81 ± 0.55	0.0009	9.09 ± 1.96	13.88 ± 1.91	0.0111
Males						
	T-turn			T-total		
Week	Sil1 ^{ht}	Sil1 ^{wz}		Sil1 ^{ht}	Sil1 ^{wz}	
14	2.17 ± 0.31	11.72 ± 3.76	0.0086	7.23 ± 1.05	20.04 ± 4.22	0.0086
16	1.89 ± 0.24	5.95 ± 0.77	0.0086	6.35 ± 0.58	15.32 ± 1.76	0.0086
Females						

<i>T-turn</i>				<i>T-total</i>		
Week	<i>Sil1^{ht}</i>	<i>Sil1^{wz}</i>		<i>Sil1^{ht}</i>	<i>Sil1^{wz}</i>	
14	4.45 ± 1.86	3.38 ± 0.28	0.0589	12.06 ± 6.92	8.00 ± 0.61	0.2466
16	3.37 ± 1.49	3.67 ± 0.45	0.1378	11.36 ± 3.41	12.44 ± 3.48	0.6191

<i>Sil1^{ht}</i>				<i>T-total</i>		
Week	Males	Females		Males	Females	
14	2.17 ± 0.31	4.45 ± 1.86	0.4326	7.23 ± 1.05	12.06 ± 6.92	0.5368
16	1.89 ± 0.24	3.37 ± 1.49	0.6623	6.35 ± 0.58	11.36 ± 3.41	0.1577

<i>Sil1^{wz}</i>				<i>T-total</i>		
Week	Males	Females		Males	Females	
14	11.72 ± 3.76	3.38 ± 0.28	0.0043	20.04 ± 4.22	8.00 ± 0.61	0.0043
16	5.95 ± 0.77	3.67 ± 0.45	0.0455	15.32 ± 1.76	12.44 ± 3.48	0.2403

Table 9 Pole test results observed during the 14th and 16th weeks. Data are reported as group mean (s) ± SEM, obtained by three trials of each animal. Significant results are highlighted in bold (Mann-Whitney test with Holm-Sidak correction for multiple comparisons). UNPUBLISHED DATA

In order to measure muscular coordination and strength, we performed the inverted grid test on mice at 16 weeks old. No significant difference in the time taken to fall from the inverted grid was observed between the *Sil1^{wz}* and *Sil1^{ht}* mice (Figure 29A, B and C). However, the *Sil1^{wz}* group spent more time without moving compared to controls, and this effect was consistent in both sexes (Figure 29G, H and I). In addition, also the number of episodes in which animals stopped moving was significantly increased in the *Sil1^{wz}* compared to the *Sil1^{ht}*, and this was evident also in the male population (Figure 29D, E and F).

Interestingly, control mice showed a significant increase in the number of times they left the grid with their hindlimbs while maintaining forelimb grip compared to *Sil1^{wz}* mice, although this difference did not remain statistically significant after separating the data by sex (Figure 29D, E and F).

No significant differences were observed between males and females within the same genotype for any of the analysed parameters (Figure 29J-M). Detailed data for this experiment are reported in Table 10.

We assumed that the total time before the animals let go of the grid indicated their muscle strength, while the duration of immobility and the frequency with which they lost grip with their hindlimbs suggested their level of physical endurance. Thus, the observed increase in immobility and the altered climbing behaviour in the woozy mice could be considered as early manifestations of muscle degeneration, despite molecular alterations in the glycolytic muscles of *Sil1^{wz}* mice are not yet detectable at this developmental stage [14].

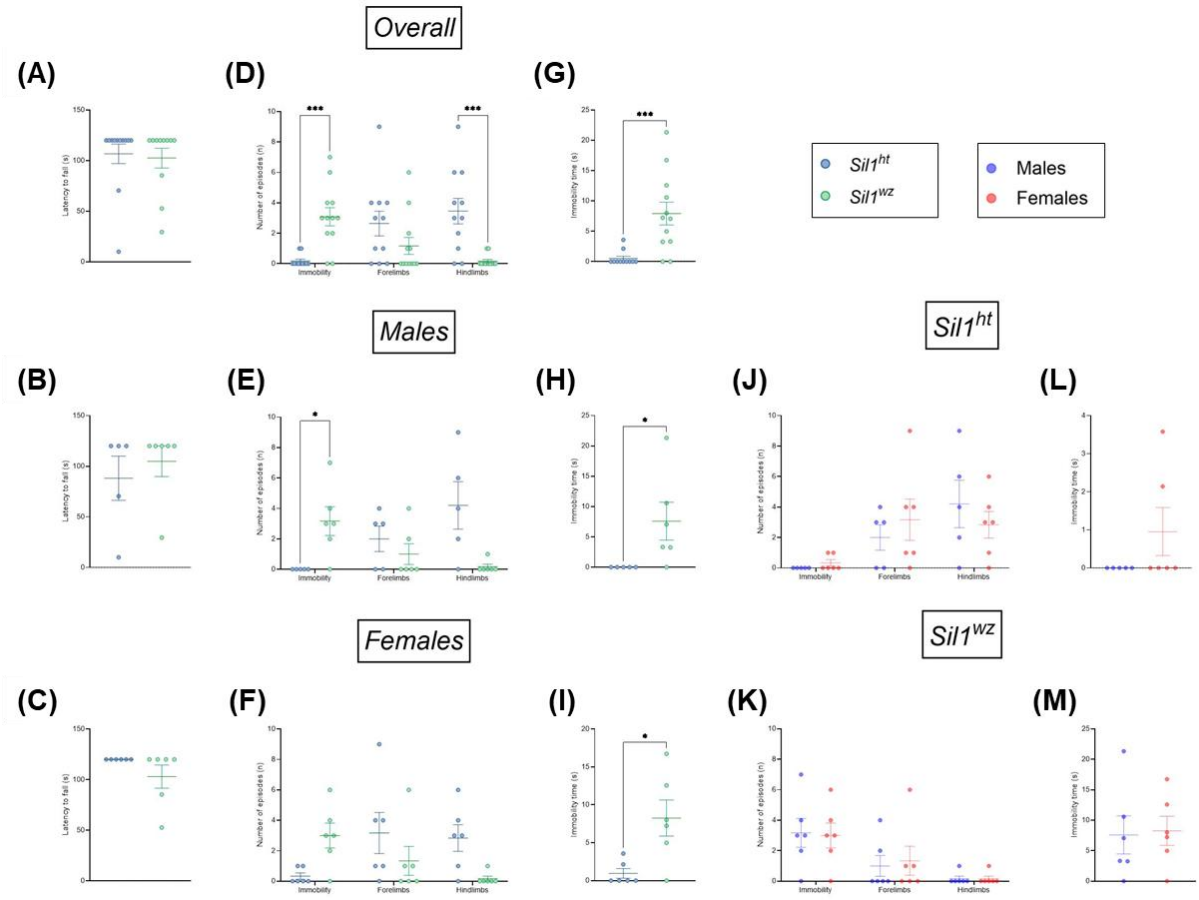


Figure 29 Inverted grid test results – Latency to fall, immobility time, and number of episodes for the individual parameter scored in the overall population (A,D,G), male (B,E,H), female (C,F,I), Sil1^{ht} (J,L), and Sil1^{wz} mice (K,M). Data are represented as scattered dot plots (mean $\pm$ SEM, of each group). Sil1^{wz} = green circles; Sil1^{ht} = blue circles; Males = blue circles; Females = red circles. Significant differences are indicated (* $p < 0.05$, *** $p < 0.005$). UNPUBLISHED DATA

		Time (s)						
Mean (s ± SEM)		p value	Mean (s ± SEM)		p value			
Overall								
Latency to fall (s)			Immobility (s)					
Sil1 ^{ht}	Sil1 ^{wz}		Sil1 ^{ht}	Sil1 ^{wz}				
106.70 ± 9.70	102.50 ± 9.80	0.06476	9.87 ± 3.72	14.02 ± 2.72	0.0129			
Males								
Sil1 ^{ht}	Sil1 ^{wz}		Sil1 ^{ht}	Sil1 ^{wz}				
88.11 ± 21.74	104.90 ± 15.09	0.4242	0.00 ± 0.00	7.59 ± 3.12	0.0152			
Females								
Sil1 ^{ht}	Sil1 ^{wz}		Sil1 ^{ht}	Sil1 ^{wz}				
103.00 ± 11.54	120.00 ± 0.00	0.4545	0.95 ± 0.64	8.26 ± 2.38	0.0216			
Sil1 ^{ht}								
Males	Females		Males	Females				
88.11 ± 21.74	103.00 ± 11.54	0.1818	0.00 ± 0.00	0.95 ± 0.64	0.4545			
Sil1 ^{wz}								
Males	Females		Males	Females				
104.90 ± 15.09	120.00 ± 0.00	>0.9999	7.59 ± 3.12	8.26 ± 2.38	0.6234			
Time (n)								
Mean (n ± SEM)		p value	Mean (n ± SEM)		p value			
Overall								
Immobility (n)			Forelimbs (n)		Hindlimbs (n)			
Sil1 ^{ht}	Sil1 ^{wz}		Sil1 ^{ht}	Sil1 ^{wz}	Sil1 ^{ht}	Sil1 ^{wz}		
0.18 ± 0.12	3.08 ± 0.60	0.0007	2.64 ± 0.81	1.17 ± 0.56	0.1221	3.46 ± 0.84	0.17 ± 0.11	0.0009

Males								
<i>Sil1^{ht}</i>	<i>Sil1^{wz}</i>		<i>Sil1^{ht}</i>	<i>Sil1^{wz}</i>		<i>Sil1^{ht}</i>	<i>Sil1^{wz}</i>	
0.00 ± 0.00	3.17 ± 0.95	0.0448	2.00 ± 0.84	1.00 ± 0.68	0.4048	4.20 ± 1.56	1.17 ± 0.17	0.0555
Females								
<i>Sil1^{ht}</i>	<i>Sil1^{wz}</i>		<i>Sil1^{ht}</i>	<i>Sil1^{wz}</i>		<i>Sil1^{ht}</i>	<i>Sil1^{wz}</i>	
3.00 ± 0.82	0.33 ± 0.21	0.0635	3.17 ± 1.35	1.33 ± 0.96	0.2619	2.83 ± 0.87	0.17 ± 0.17	0.0635
<i>Sil1^{ht}</i>								
Males	Females		Males	Females		Males	Females	
0.00 ± 0.00	3.00 ± 0.82	0.8377	2.00 ± 0.84	3.17 ± 1.35	0.8377	4.20 ± 1.56	2.83 ± 0.87	0.8377
<i>Sil1^{wz}</i>								
Males	Females		Males	Females		Males	Females	
3.17 ± 0.95	0.33 ± 0.21	0.9995	1.00 ± 0.68	1.33 ± 0.96	0.9965	1.17 ± 0.17	0.17 ± 0.17	>0.9999

Table 10 Inverted Grid test results observed during the 16th week. Data are reported as group mean ± SEM, obtained by three trials of each animal. Significant results are highlighted in bold (Mann-Whitney test with Holm-Sidak correction for multiple comparison). UNPUBLISHED DATA

3.4.2 Assessment of Cognitive Behaviour in The *Sil1^{wz}* Mouse Model

To explore potential cognitive deficits in *Sil1^{wz}* mouse model, given the frequent intellectual disability in MSS patients, we conducted three different behavioural tests.

Firstly, we investigated the nesting building ability of *Sil1^{wz}* compared to *Sil1^{ht}* mice at their 10th and 14th week of life, through the evaluation of three parameters: paper movement in the cage compared to the original place, folds opening, and gnawing percentage of the entire layer (see Methods section).

At 10 weeks, no significant difference in the nesting score was observed between the two groups. However, by 14 weeks, *Sil1^{wz}* mice showed a significant decrease in the total nesting score compared to *Sil1^{ht}* mice (Figure 30A), particularly because of the lower percentage of paper gnawing.

We did not observe a significant influence of sex on these results (Figure 30B and 30C), nor significant differences when comparing males and females within the same genotype (Figure 30D and E). Complete data of this test are reported in Table 11.

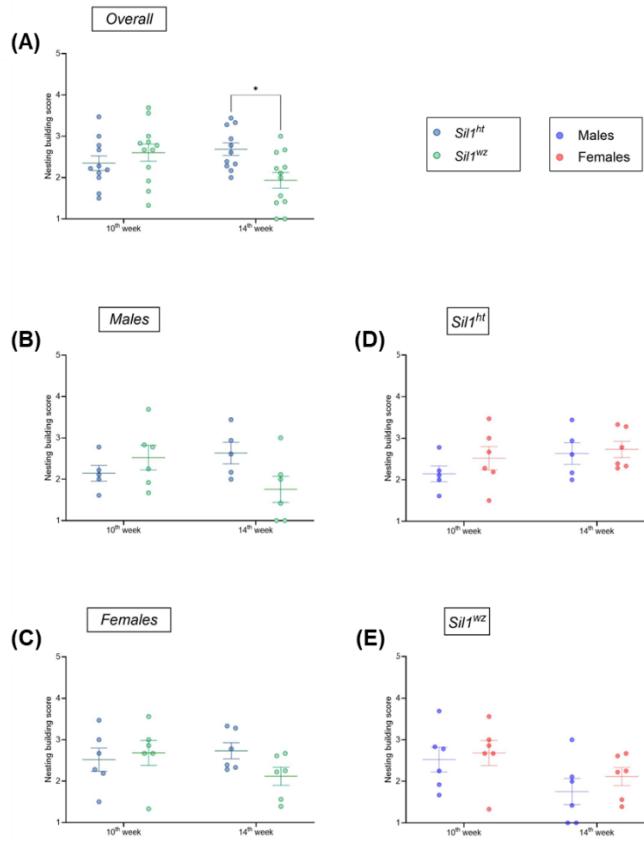


Figure 30 Nesting Building results – Average score of the paper movement, degree of opening, and gnawing percentage at the 10th and 14th week of life in the overall population (A), male (B), female (C), *Sil1^{ht}* (D), and *Sil1^{wz}* mice (E). Data are represented as scattered dot plots (mean $\pm$ SEM, of each group). *Sil1^{wz}* = green circles; *Sil1^{ht}* = blue circles; Males = blue circles; Females = red circles. Significant differences are indicated (* $p < 0.05$). UNPUBLISHED DATA

	Mean (s ± SEM)			p value	Mean (s ± SEM)			p value	Mean (s ± SEM)			p value		
	Movement				Opening				Gnawing				Total	
Overall	Week	<i>Sil1^{ht}</i>	<i>Sil1^{wz}</i>		<i>Sil1^{ht}</i>	<i>Sil1^{wz}</i>		<i>Sil1^{ht}</i>	<i>Sil1^{wz}</i>		<i>Sil1^{ht}</i>	<i>Sil1^{wz}</i>		
	10	2.64 ± 0.38	3.41 ± 0.38	0.3876	1.71 ± 0.15	1.37 ± 0.19	0.3404	2.70 ± 0.36	3.03 ± 0.41	0.5413	2.35 ± 0.18	2.60 ± 0.20	0.3084	
	14	2.92 ± 0.39	2.43 ± 0.33	0.5413	2.06 ± 0.14	1.58 ± 0.16	0.3027	3.08 ± 0.25	1.79 ± 0.25	0.0120	2.69 ± 0.15	1.94 ± 0.19	0.0199	
Males	Week	<i>Sil1^{ht}</i>	<i>Sil1^{wz}</i>		<i>Sil1^{ht}</i>	<i>Sil1^{wz}</i>		<i>Sil1^{ht}</i>	<i>Sil1^{wz}</i>		<i>Sil1^{ht}</i>	<i>Sil1^{wz}</i>		
	10	3.10 ± 0.66	3.92 ± 0.35	0.7821	1.60 ± 0.27	1.21 ± 0.14	0.7236	1.73 ± 0.32	2.44 ± 0.66	0.7822	2.14 ± 0.19	2.52 ± 0.30	0.3528	
	14	3.23 ± 0.62	2.46 ± 0.55	0.7823	2.00 ± 0.21	1.33 ± 0.21	0.2639	2.67 ± 0.38	1.47 ± 0.33	0.2685	2.63 ± 0.26	1.76 ± 0.32	0.1814	
Females	Week	<i>Sil1^{ht}</i>	<i>Sil1^{wz}</i>		<i>Sil1^{ht}</i>	<i>Sil1^{wz}</i>		<i>Sil1^{ht}</i>	<i>Sil1^{wz}</i>		<i>Sil1^{ht}</i>	<i>Sil1^{wz}</i>		
	10	2.25 ± 0.40	2.90 ± 0.63	0.9488	1.81 ± 0.19	1.53 ± 0.35	0.9488	3.50 ± 0.37	3.61 ± 0.40	0.9488	2.52 ± 0.28	2.68 ± 0.30	0.6147	
	14	2.67 ± 0.52	2.40 ± 0.41	0.9488	2.11 ± 0.20	1.83 ± 0.21	0.9488	3.42 ± 0.28	2.11 ± 0.34	0.0875	2.73 ± 0.20	2.12 ± 0.22	0.1257	
<i>Sil1^{ht}</i>	Week	Males	Females		Males	Females		Males	Females		Males	Females		
	10	3.10 ± 0.66	2.25 ± 0.40	0.8885	1.60 ± 0.27	1.81 ± 0.19	0.9368	1.73 ± 0.32	3.50 ± 0.37	0.0508	2.14 ± 0.19	2.52 ± 0.28	0.5498	
	14	3.23 ± 0.62	2.67 ± 0.52	0.9368	2.00 ± 0.21	2.11 ± 0.20	0.9368	2.67 ± 0.38	3.42 ± 0.28	0.5822	2.63 ± 0.26	2.73 ± 0.20	0.7922	
<i>Sil1^{wz}</i>	Week	Males	Females		Males	Females		Males	Females		Males	Females		
	10	3.92 ± 0.35	2.90 ± 0.63	0.7527	1.21 ± 0.14	1.53 ± 0.35	0.9081	2.44 ± 0.66	3.61 ± 0.40	0.6477	2.52 ± 0.30	2.68 ± 0.30	0.6688	
	14	2.46 ± 0.55	2.40 ± 0.41	0.9935	1.33 ± 0.21	1.83 ± 0.21	0.6477	1.47 ± 0.33	2.11 ± 0.34	0.4109	1.76 ± 0.32	2.12 ± 0.22	0.4960	

Table 11 Nesting building test results observed during the 10th and 14th weeks. Data are reported as group mean (s) $\pm$ SEM. Significant results are highlighted in bold (Mann-Whitney test with Holm-Sidak correction for multiple comparisons). UNPUBLISHED DATA

Secondly, we performed the novel object recognition test on *Sil1^{wz}* and *Sil1^{ht}* at the 5th and at the 14th week of age (see Methods section). The evaluation was based on the time spent exploring the familiar and the novel objects.

This test didn't show significant differences between the two groups at either the 5th or the 14th week, even when analysing the data based on sex or genotype (Table 12).

Mean (s ± SEM)			p value
Overall			
Discrimination index (%)			
Week	<i>Sil1^{ht}</i>	<i>Sil1^{wz}</i>	
5	66.03 ± 5.02	59.08 ± 7.06	0.7780
14	66.48 ± 7.67	62.53 ± 8.07	0.7780
Males			
T-turn			
Week	<i>Sil1^{ht}</i>	<i>Sil1^{wz}</i>	
5	64.50 ± 8.51	53.78 ± 6.90	0.5682
14	81.63 ± 5.29	73.43 ± 7.72	0.5682
Females			
T-turn			
Week	<i>Sil1^{ht}</i>	<i>Sil1^{wz}</i>	
5	68.34 ± 2.29	67.01 ± 14.81	0.5682
14	56.38 ± 10.68	55.27 ± 12.06	0.9372
<i>Sil1^{ht}</i>			
T-turn			
Week	<i>Males</i>	<i>Females</i>	
5	64.50 ± 8.51	68.34 ± 2.29	>0.9999
14	81.63 ± 5.29	56.38 ± 10.68	0.4482
<i>Sil1^{wz}</i>			
T-turn			
Week	<i>Males</i>	<i>Females</i>	
5	53.78 ± 6.90	67.01 ± 14.81	0.4482
14	73.43 ± 7.72	55.27 ± 12.06	0.4482

Table 12 Novel object recognition test results observed during the 5th and 14th weeks. Data are reported as group mean (s) $\pm$ SEM, obtained by three trials of each animal. UNPUBLISHED DATA

Similarly, the Y-maze test, which is able to monitor the working memory and the explorative ability, didn't reveal significant differences between *Sil1^{wz}* and *Sil1^{ht}* at the 5th and at the 14th week of life. Moreover, we were not able to observe differences between the groups also when looking at sex-based or genotype-based stratification of the data. All the group means, and *p*-values are reported in Table 13.

Mean (s $\pm$ SEM)			p value
<i>Overall</i>			
Arms alternation (%)			
Week	<i>Sil1^{ht}</i>	<i>Sil1^{wz}</i>	
5	68.00 $\pm$ 2.62	69.60 $\pm$ 4.20	0.9639
14	66.00 $\pm$ 3.06	67.30 $\pm$ 2.93	0.9639
<i>Males</i>			
<i>T-turn</i>			

Week	<i>Sil1^{ht}</i>	<i>Sil1^{wz}</i>	
5	63.67 ± 3.03	66.17 ± 5.14	0.6753
14	62.25 ± 5.54	70.25 ± 1.84	0.5298
Females			
<i>T-turn</i>			
Week	<i>Sil1^{ht}</i>	<i>Sil1^{wz}</i>	
5	74.50 ± 2.18	74.75 ± 7.18	>0.9999
14	68.50 ± 3.56	65.33 ± 4.73	0.9095
<i>Sil1^{ht}</i>			
<i>T-turn</i>			
Week	Males	Females	
5	63.67 ± 3.03	74.50 ± 2.18	0.1289
14	62.25 ± 5.54	68.50 ± 3.56	0.6857
<i>Sil1^{wz}</i>			
<i>T-turn</i>			
Week	Males	Females	
5	66.17 ± 5.14	74.75 ± 7.18	0.6109
14	70.25 ± 1.84	65.33 ± 4.73	0.6109

Table 13 Y-maze test results observed during the 5th and 14th weeks. Data are reported as group mean (s) ± SEM, obtained by three trials of each animal. UNPUBLISHED DATA

3.4.3 Molecular Analysis of The Quadriceps and Soleus Muscles in The *Sil1^{wz}* Mouse Model

Finally, we focused on the molecular study of *Sil1^{wz}* mouse muscles at their 26 weeks of age. In particular, we validated the expression of previously identified markers in the quadriceps muscle [14], and investigated the molecular differences between this glycolytic muscle and the soleus, which has an oxidative metabolism.

Through Western blot analysis, we confirmed the upregulation of proteins associated with the UPR, including BiP, PdI, peIF2 α and Chop, as well as proteins involved in proteolysis, such as Rab11 and lipidated form of LC3 (LC3-II) in the quadriceps of *Sil1^{wz}* mice compared to controls (*Sil1^{ht}*) [14]. Conversely, levels of LC3-I and Creatine kinase B (CKB) were decreased (Figure 31A and B).

When we examined the same markers in the soleus muscle of the same mice, we noted a slight increase in BiP and Rab11, along with a decrease in CHOP, but in the other investigated proteins no significant differences were found (Figure 31A and C). In this analysis, sex-based stratification of the data was not relevant (data not shown).

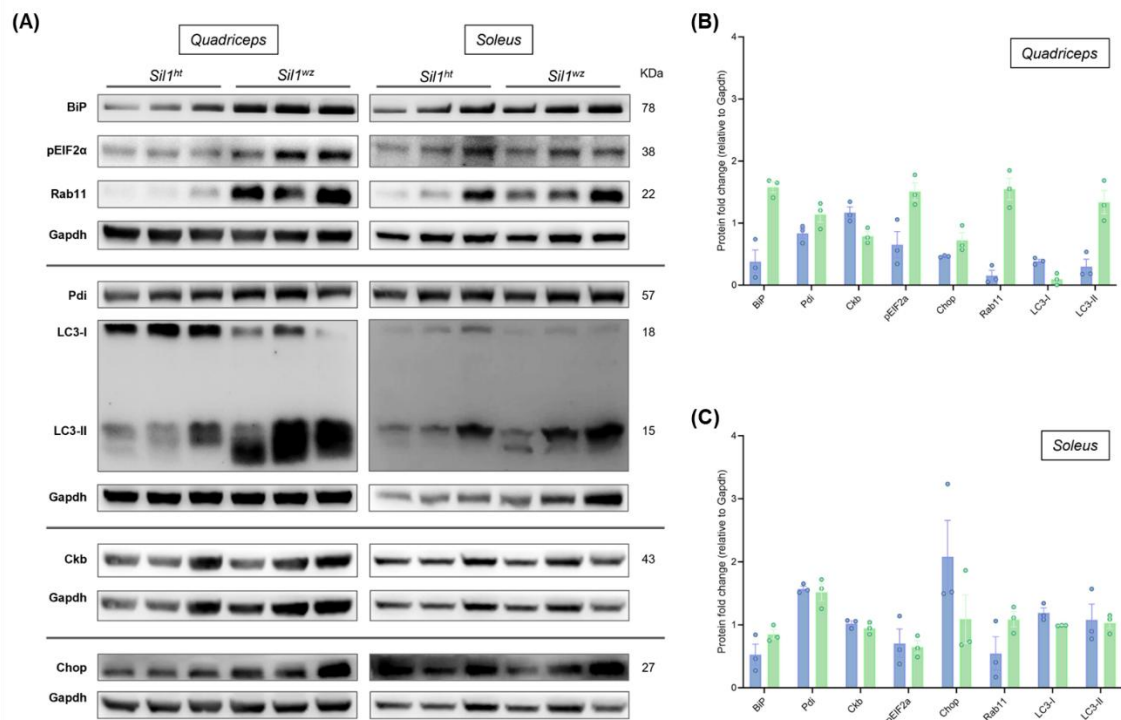


Figure 31 Western Blot analysis performed in 26-week-old *Sil1^{ht}* and *Sil1^{wz}* mice (a). Protein fold change (relative to Gapdh) observed in the quadriceps (b) and soleus (c) of *Sil1^{ht}* and *Sil1^{wz}* mice. UNPUBLISHED DATA

In conclusion, these findings represent our effort to provide a better characterization of the woozy mouse model, to pave the way for the development of potential approaches for MSS. Through motor testing and molecular approaches, we observed muscular deficits mirroring those in human patients, beyond the expected cerebellar damage. In addition, we introduced a preliminary cognitive characterization of *Sil1^{wz}* mice. However, further studies are necessary to investigate the influence of sex and hormones in this model, and the differential mechanisms in affected and healthy tissues.

4. Discussion

MSS is an early-onset autosomal recessive disorder with varying degrees of severity, typically characterized by ataxia, myopathy, intellectual disability and several skeletal and eye problems [15, 18, 22]. In about 60% of cases, this condition arises from loss-of-function mutations of Sil1, a widely expressed cochaperone located in the endoplasmic reticulum (ER) [11]. However, consistent with the current understanding of MSS, a clear genotype-phenotype correlation remains elusive due to the variable clinical presentation among patients and the increasing identification of diverse *SIL1* sequence variants [54, 55]. The absence of functional Sil1 in individuals with MSS leads to a disruption of ER homeostasis, which results in UPR activation. Interestingly, despite UPR is likely involved in the pathogenesis of MSS and Sil1 is a ubiquitous protein, it is still not clear the reason why certain tissues undergo degeneration (Purkinje cells and muscle fibers) while others survive [18]. Thus, we decided to investigate the molecular mechanisms underlying MSS by studying the impact of mutation of Sil1 *in vitro* and *in vivo*.

For this purpose, we first evaluated both the protein and the gene expression profiles of skin fibroblasts obtained from an MSS patient affected by the *SIL1* R111X mutation, providing considerable understanding of the molecular consequences of Sil1 loss in these cells.

Prompted by our recent findings, we next conducted an in-depth study of the new *SIL1* E342K variant in order to establish its pathogenicity for MSS. By comparing the molecular markers revealed in the first patient with the second one, we found significant similarities. Interestingly, these results suggested a conservation of the molecular phenotype.

Finally, we looked at the impact of *SIL1* mutation on the animal model of *woozy* mouse, specifically investigating the development of muscular deficits, alterations in cerebellar functions, and the presence of potential cognitive impairment.

Our detailed study was aimed at shedding light on the pathogenesis of MSS and discovering new relevant markers useful in the development of possible new therapies.

The key findings of our research are discussed below.

4.1 Sil1 Loss in MSS Fibroblasts Triggers Pro-Survival Metabolic Adaptation

Despite not being a primary target of MSS, fibroblasts derived from an MSS patient carrying the *SIL1* R111X mutation (HF-MSS) showed a mild UPR activation, along with the upregulation of endolysosomal markers. Our proteomic analysis identified over 600 differentially expressed proteins in these cells compared to healthy fibroblasts, highlighting a downregulation of RNA splicing and transport machinery, ribosomes and translation initiation [21]. These changes could be due to the protein synthesis reduction mediated by the UPR, but the downregulation of the spliceosome was unexpected. Notably, these alterations were considered as survival mechanisms in several tumor cell lines treated with various anticancer agents and in senescent fibroblasts [56, 57]. In addition, HF-MSS cells exhibited other markers of senescence, such as reduced levels of histone proteins and increased size, with a lower proliferation rate [21, 58, 59]. Metabolically, MSS fibroblasts experienced significant metabolic reprogramming to face Sil1 loss. In particular, the catabolism of fatty acids and amino acids was increased, along with a boost of TCA cycle and the upregulation of mitochondrial ATP synthase [21].

These alterations may be attributed to increased mitochondrial ATP demand under ER stress conditions, in order to improve protein folding and trafficking while restoring ion homeostasis [60]. A graphical representation of the proteomics results is shown in Figure 32.

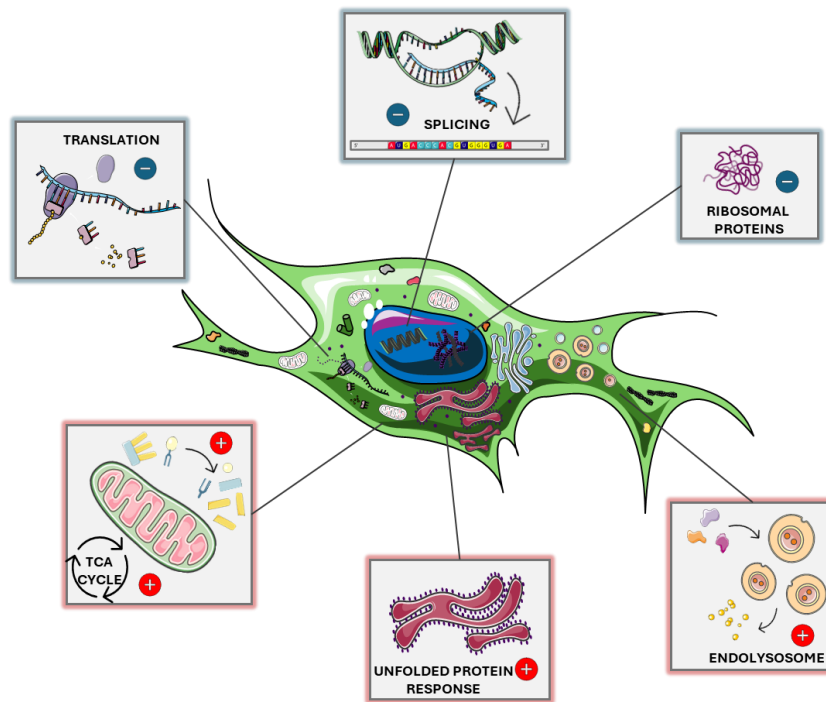


Figure 32 Marinesco-Sjögren fibroblast showing mild signs of UPR. Proteomic analysis of Marinesco-Sjögren fibroblasts revealed downregulation in processes such as splicing, ribosome biogenesis, and translation. In contrast, the TCA cycle is upregulated due to increased acetyl-CoA production, which results from enhanced protein catabolism via lysosome-based structures. Additionally, biosynthesis of certain amino acids is reduced, lipid production is downregulated, and beta-oxidation is increased.

From “Genotype-phenotype correlation and adaptive proteome reorganization in Marinesco-Sjögren syndrome. Ruggieri and Sallese, 2022. DOI: 10.36253/ijae-13802.

4.2 *Sil1* Deficiency in Fibroblasts Disrupts ECM Homeostasis, Resulting in Tendon Disorganisation in MSS

The analysis of the gene expression profile of the same MSS fibroblasts revealed over 600 altered transcripts compared to control cells [41], which exhibited a strong correlation with previous proteomic findings [21, 61].

Data analysis revealed alterations in cellular organelles and ECM formation and remodelling, as indicated by changes in collagens, glycosaminoglycans, metalloproteases and their inhibitors, along with cytoskeleton and cell adhesions.

Through *in vitro* functional studies, we confirmed the impairment of ECM remodelling and matrix internalisation and provided evidence of affected attachment and motility of MSS fibroblasts. These *in vitro* findings were corroborated by TEM analysis of the myotendinous junction of the *woozy* mouse model, which showed disrupted collagen fibrils organisation and ECM degeneration, despite *SIL1* mutations in the patient and in the mouse model being

different. Key results of this study are summarised in Figure 33.

It is known that ECM is crucial for neuronal functioning [62], and its alterations are implicated in neurodegenerative diseases including Alzheimer's disease, Huntington's disease (HD), Parkinson's disease (PD), epilepsy and ataxia [63, 64]. Furthermore, ECM is vital for muscle physiology and regeneration [65], and its disruption often leads to myopathies [66, 67]. In the eye, ECM maintains tissue structure and functions, and its disturbances contribute to conditions like cataracts, potentially linked to versican overexpression and ALDH1A1 downregulation as observed in MSS [68, 69, 70]. In addition, alterations in cartilage ECM, especially in biglycan and aggrecan which were also altered in our transcriptomics, are associated with chondrodysplasias [71, 72].

In conclusion, given all these findings, our hypothesis is that a dysfunctional ECM is a central pathological hallmark of MSS and could provide insights into the majority of its clinical symptoms.

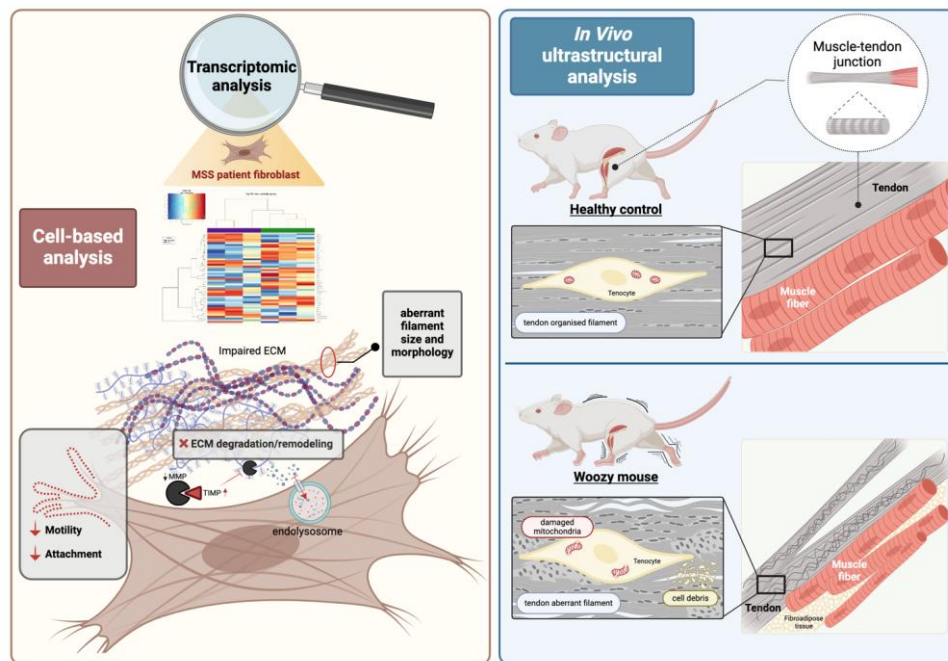


Figure 33 Molecular, cellular, and histological analysis of ECM and tendon alterations in woozy mice. *In vitro* and *in vivo* approaches were used to uncover changes in the extracellular matrix (ECM) and tendons of woozy mice, which may contribute to the pathological mechanisms of MSS. The left panel summarises *in vitro* findings, highlighting genes differentially expressed in control and patient fibroblasts. These molecular changes disrupt ECM dynamics, leading to an aberrant ECM that impairs cell adhesion and reduces cell motility. The right panel shows disorganised fibrils in the woozy mouse tendon, both histologically and ultrastructurally, along with evidence of tenocyte damage.

From "Sil1-deficient fibroblasts generate an aberrant extracellular matrix leading to tendon disorganisation in Marinesco-Sjögren syndrome" Amodei et al., 2024. DOI: 10.1186/s12967-024-05582-0.

4.3 Impact of The Novel P.E342K *SIL1* Variant on Protein Structure and Function in MSS

We carried out a detailed molecular analysis to investigate the pathogenicity of the novel c.1024G>A variant of unknown significance, discovered in a young Greek patient presenting with MSS-like symptoms. The resultant mutant Sil1 protein with a lysine at position 342 instead of a glutamic acid showed an impaired structure due to the altered network of interactions in the middle of the armadillo repeats area, and to the reduction of the alpha-helical organisation. *In vitro* experiments revealed that Sil1 p.E342K had poor solubility, resembling the known pathological behaviour of Sil1 p.L457P [46, 47]. Furthermore, patient-derived fibroblasts (HF-P2) confirmed a dysregulation of 10 out of 12 genes previously identified as an MSS signature in fibroblasts carrying the *SIL1* R111X mutation [41]. However, the changes were less pronounced, suggesting that the p.E342K variant might be a milder variant.

HF-P2 cells showed increased Sil1 mRNA but reduced protein levels, suggesting mutant protein instability and misfolding. The elevated mRNA levels may reflect a compensatory response to the reduced protein expression, while the reduced protein levels depend on proteasomal degradation, as demonstrated with the MG132 treatment. We also observed enlarged lysosomes and accumulated lipid droplets, consistent with metabolic alterations reported in MSS [21], and also the presence of fibrotic material, which was observed in other neurodegenerative diseases [73].

The proteomic profile of HF-P2 revealed a significant overlap in altered protein expression with HF-P1 fibroblasts and the muscle tissue of an MSS mouse model, reinforcing the pathological nature of p.E342K [21, 25].

Collectively, our molecular, biochemical, and cellular data strongly support the conclusion that the novel c.1024G>A (p.E342K) *SIL1* variant is pathogenic and causes MSS, despite appearing less severe at the cellular level than the p.R111X nonsense mutation.

A graphical summary of this work is shown in Figure 34.

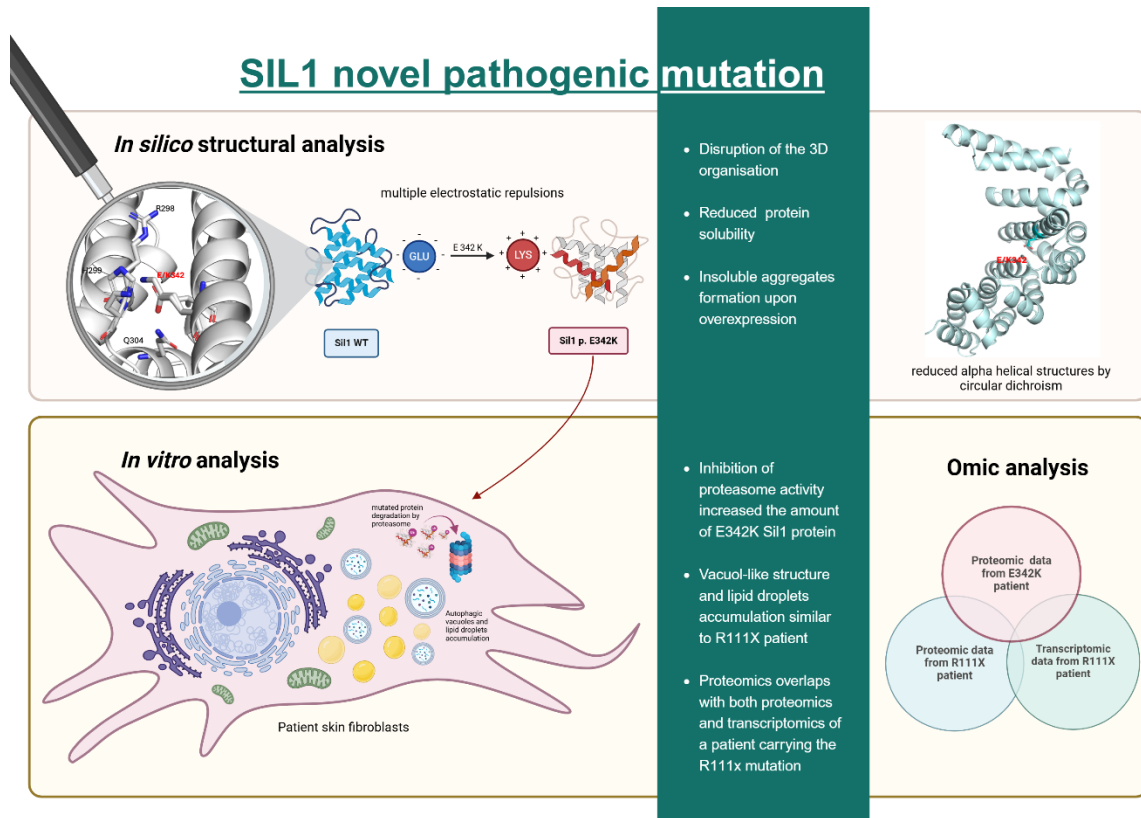


Figure 34 *SIL1* novel pathogenic mutation investigation through several approaches. *In silico* structural analysis highlighted the impact of the amino acid substitution on the stability of the protein. *In vitro* analysis revealed and proteomics confirmed respectively the MSS phenotype and its molecular profile. UNPUBLISHED DATA

4.4 *Sil1* loss in the wooly mouse model

To uncover further details regarding the *Sil1*^{wz} mouse model that have not been reported previously [14, 24, 48, 49, 50], we performed a broader phenotypic characterisation, specifically analysing the contribution of sex. In addition to the motor impairment, we investigated the presence of possible cognitive deficits through a series of tests. Moreover, we conducted a comparative molecular analysis of the mouse muscles, examining the differences between a glycolytic and an oxidative muscle type, to confirm our *Sil1*^{wz} mouse model.

Rotarod test confirmed prior findings of cerebellar dysfunction [24], [50], with *Sil1*^{wz} mice showing decreased rotarod latency from the 9th week, observing generally worse male performance on the rotarod and a delayed onset of deficits in female *Sil1*^w mice.

In comparison with our results, Grande and colleagues started observing differences between *Sil1*^{wz} and *Sil1*^{ht} from one week before (8th week of life) [24]. This could be due to the inclusion of wild-type and *Sil1*^{ht} mice in the control group. However, the *Sil1*^{ht} and wild-type animal groups should not exhibit any differences in both motor performance and molecular features

[24]. On the contrary, the delayed onset of decreased latency time observed by Hayashi et al. (11th week of life) was probably caused by the use of a different protocol in the accelerating rotarod test [50].

Next, the beam walking test, which is also specific for monitoring coordination and balance [52, 53], revealed earlier and more pronounced coordination deficits in *Sil1^{wz}* mice, with males affected sooner than females, although sex did not influence the number of foot slips. Notably, this test is more sensitive than rotarod in evaluating motor deficits, as previously revealed [24]. The pole test, despite its experimental simplicity, is a challenging task, and performance can be influenced by diverse neuronal dysfunctions. For this reason, it has been considered an effective tool for the evaluation of anti-Parkinsonian drugs efficacy [74, 75, 76]. Our results highlighted cerebellar dysfunction in *Sil1^{wz}* mice through increased turning time, particularly evident after significant Purkinje cell loss, with females performing better than males.

Furthermore, the inverted grid test, which is useful for the evaluation of neuromuscular functions by measuring muscle coordination and strength [53, 77], showed no difference in fall latency but revealed reduced movement and hindlimb detachment. We hypothesised that this behaviour could represent the initial “visible” signs of muscle impairment in *Sil1^{wz}* mice before the onset of molecular alterations in their glycolytic muscles [14].

To investigate a potential cognitive impairment in *Sil1^{wz}* mice, considering the mental disability present in most MSS patients [78], we performed nesting, novel object recognition (NOR), and Y-maze tests. These tests revealed a specific deficit only in nesting behaviour in *Sil1^{wz}* mice at 14 weeks, with females showing better nesting abilities overall [79]. Particularly, the nest-building test is a simple, non-invasive behavioural assay that provides information about general health, along with cognitive and motor functions [80, 81], and it has already been used to study other neurological disorders [82, 83]. Y-maze test is usually used for the assessment of spatial working memory [84, 85], but in this case it didn’t highlight significant differences between *Sil1^{wz}* and *Sil1^{ht}* mice. We could hypothesise that *Sil1^{wz}* mice don’t have an impairment in spatial working memory. Similarly, the NOR test [86, 87] didn’t reveal particular learning and memory deficits, thus probably excluding an involvement of the hippocampus in the neurodegenerative process of these mice, due to its role in the formation of the working memory [88].

Overall, our observed results confirmed motor impairments through the use of other novel tests, and suggested a possible cognitive deficit due to the altered nesting behaviour. However, deeper investigations are needed to reveal if other brain structures could be affected by *Sil1* loss,

causing cognitive disabilities.

Finally, our molecular analysis of quadriceps muscle in *Sil1^{wz}* mice confirmed increased UPR and other markers previously reported [14], while these changes were less evident in the oxidative soleus muscle, which is known to be less affected in MSS [25].

In conclusion, our comprehensive analysis displayed the impact of *Sil1* loss on the *woozy* mouse, particularly on the cerebellum and the muscle tissue, while also revealing notable sex-specific differences.

5. Conclusions

Our research was aimed at a deeper understanding of the molecular mechanisms underlying the Marinesco-Sjögren Syndrome and resulting from the lack of Sil1 *in vitro* and *in vivo*.

Although fibroblasts are not the main target of the pathology, these cells have proven to be a helpful system for studying cellular responses to Sil1 deficiency, and also for the evaluation of the pathogenicity of a new VUS. However, an *in vitro* model representative of the mainly affected cells is still lacking.

In this scenario, the woozy mouse model exhibited its utility for studying MSS, through the assessment of motor and cognitive deficits. Further studies on animal models could provide a deeper insight into the MSS pathology and contribute to the discovery of novel biomarkers in order to monitor the disease progression and develop effective new treatments.

6. Methods

6.1 *Methods of Proteomic Analysis of Marinesco-Sjögren Syndrome Fibroblasts*

6.1.1 *Reagents*

The following primary antibodies were used for WB as indicated: SIL1 (Proteintech #24110-1-AP); ATF4 (Cell Signaling #11815); ATF6B (Proteintech #15794-1-AP); pho-Elf2 α (Cell Signaling #3597s); PDI (Assay Design #SPA-891); BiP (BD#610979); ORP150 (Abcam #EP5891); LC3B (Cell Signaling #2775); NCL (Cell Signaling #87792S); NPM (Cell Signaling #3542S); CTHRC1 (Abcam #ab85739); Anti SQSTM1 (BD Bioscience #610833); LAMP1 (Abcam #ab25630); CNX (Cell Signaling #2679S); NES (Millipore #AB5922); CAV-1 (Cell Signaling #3238); and GAPDH (Santa Cruz #sc-32233). For PCR amplification of the spliced and unspliced form of human XBP1, the following primers were used:

FW 5'-AGCTCAGACTGCCAGAGATCG-3';

REV 5'-AATCCATGGGGAGATGTTCTA-3'.

Unless specified otherwise, all powders, buffers, and other routine reagents were supplied Sigma-Aldrich and were of analytical grade or higher [21].

6.1.2 *Cell culture*

Primary human dermal fibroblasts (NDHF Promo Cell #FB60C12350) supplied by Carlo Erba were used as the control cell line and compared to primary dermal fibroblasts derived from a young patient affected by Marinesco–Sjögren syndrome (supplied by Telethon Network of Genetic Biobanks—TNGB [89]). Cells were cultured in Dulbecco's modified Eagle's medium + GlutaMAX (GIBCO, 61965-026), supplemented with 10% of Fetal Bovine Serum (FBS) and 1% penicillin/streptomycin (PenStrep, GIBCO, 15070-063). Cells were maintained at 37 °C, 5% CO₂, and detached by Trypsin-EDTA 0.5% (GIBCO, 15400-054) [21].

6.1.3 *Western Blot Analysis and PCR*

Cells were lysed using RIPA buffer (tris HCl 50 mM (pH 7.6), NaCl 140 mM, EDTA 5 mM, 0.1% SDS, 1% NP40, NaF 100 mM, 0.1% Sodium deoxycholate) supplemented with protease inhibitors (cOmpleteTM Tablets, Roche) and phosphatase (PhosSTOP tablets, Roche)

inhibitors. Protein concentration was determined using the Bradford protein assay (Bio-Rad). Equal amounts of proteins were then prepared in 4x Laemmli buffer (15% b-mercaptoethanol, Tris-HCl 0.25 M pH 6.8, 40% Glycerol, 8% SDS, 0.02% Bromophenol Blue), separated by SDS-PAGE, and transferred onto nitrocellulose membrane. Protein loading and transfer efficiency were assessed by Ponceau S staining (0.5% (w/v) Ponceau S dissolved in 1% (v/v) acetic acid). Membranes were incubated for 5 min in Ponceau S solution and then washed once with water, photographed, and destained with TBS-T. Ponceau S staining was used as a Western blotting multiprotein loading control throughout the manuscript instead of a single protein (e.g., β -actin), whose expression might be altered in HF-MSS cells. Membranes were blocked with 5% non-fat dry milk or 5% BSA for 1 hour at room temperature and incubated with primary antibodies overnight at 4 °C. Secondary antibodies α -rabbit and α -mouse were applied the day after for 1 h at room temperature and signal was detected by GE Amersham ECL. For XBP1 splicing analysis by PCR [90], RNA was extracted using the miRNeasy kit (QIAGEN cat. 217004). cDNA was synthesised using a reverse transcription amplification kit (Applied Biosystems), and PCR was performed with an annealing temperature of 54 °C for 40 cycles. PCR products (10 μ L) were separated on a 3% agarose gel [21].

6.1.4 Proliferation Assay

The Click-iT Edu Proliferation assay kit (Invitrogen, #C10499) was used to monitor cell proliferation following the manufacturer's instructions. Approximately 10,000 cells were plated per well in a 96-well plate 24 hours before the experiment.

6.1.5 Label-Free Proteomics Analysis

Human fibroblasts from MSS patients (HF-MSS) were grown to subconfluency, serum-starved for 24 hours, washed, and lysed in a proteomic buffer (Urea 6 M, Tris Base 100 mM, 2% CHAPS, 1% Triton X-100, and DTT 50 mM). Protein samples were then processed using the Filter Aided Sample Preparation (FASP) method. Protein concentration was determined by Bradford assay (Bio-Rad).

For label-free quantitative proteomics, 25 μ g of protein from each sample was digested with trypsin (Sigma-Aldrich). Tryptic peptides were analysed in triplicate by LC-MS/MS using an UltiMateTM 3000 UPLC system (Thermo Fisher Scientific, Waltham, MA, USA) coupled to an Orbitrap FusionTM TribridTM mass spectrometer (Thermo Fisher Scientific). Peptides were loaded onto a C18 trap column (0.3 mm ID, 5 mm L, 5 μ m PS, Thermo Fisher Scientific) and

then separated on an EASY-spray AcclaimTM PepMapTM C18 (75 μ m ID, 25 cm L, 2 μ m PS, Thermo Fisher Scientific) nanoscale chromatographic column. The mobile phase A was 0.1% formic acid in H₂O and the mobile phase B was 0.1% formic acid in acetonitrile. The flow rate was set at 300 nL/min, with a total run time of 125 min using a chromatographic gradient from 2% to 90% of phase B.

Mass spectrometry data were acquired in positive-ion polarity using the Data Dependent Acquisition (DDA) mode to trigger precursor isolation and MS2 sequence using N₂ as the collision gas for HCD fragmentation. The positive ion voltage was set at 1700 V and the ion transfer tube at 275 °C for desolvation. MS1 scans were performed in the Orbitrap analyser covering an m/z range of 375–1500 with a resolution of 240,000. Standard automatic gain control (AGC) and automatic maximum injection time (MIT) settings were employed. The signal intensity threshold was set to 1.0×10^4 and the MS2 spectra were acquired using a Top Speed method of 3s. In particular, precursor ions with charges of +2 to +5 were used for MS2 sequencing and scanned in the ion trap by setting the following parameters: MS2 isolation window of 1.6 Da, AGC target of 1.0×10^4 , dynamic exclusion time of 60 s, and mass tolerance of ± 10 ppm were used covering an m/z range of 300–1200.

We performed a HCD fragmentation with a fixed collision energy of 30% and a MIT of 70 ms. The mass spectrometry proteomics data were deposited to the ProteomeXchange Consortium via the PRIDE partner repository [91] with the dataset identifier PXD029086 [21].

6.1.6 *Electron Microscopy (EM)*

Approximately 2×10^6 HF cells cultured in monolayers were prepared for EM as follows. Cells were detached from the dish with trypsin, centrifuged at $180 \times g$ for 2.5 min, and washed 3 times with PBS at 37 °C. Fixation was performed with 3.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (NaCaCO) for 1 hour, followed by storage at 4 °C. For embedding, cells were postfixated in 2% OsO₄ in the same buffer for 2 h and were block-stained with saturated uranyl acetate replacement. After dehydration, specimens were embedded in epoxy resin (Epon 812). Ultrathin sections were cut using a Leica Ultracut R microtome (Leica Microsystem, Austria) with a Diatome knife (Diatome Ltd. CH-2501 Biel, Switzerland) and double-stained with uranyl acetate replacement and lead citrate. Sections were examined and photographed using a Morgagni Series 268D electron microscope (FEI Company, Brno, Czech Republic) equipped with a Megaview III digital camera and Analy-SIS software (Olympus Soft Imaging Solutions). Quantitative analysis was done on random micrographs of HF cell cultures (CT and MSS). The

percentage of cells displaying cytoplasmic accumulation of membrane-bound vacuoles containing electron-dense material was determined from micrographs taken at 5600× or 7100× magnification (sample size: 100 cells per group) [21].

6.2 Methods of Transcriptomic Analysis of Marinesco-Sjögren Syndrome Fibroblasts

6.2.1 Cell cultures

Primary human dermal fibroblasts were used as the control cell line and compared to primary dermal fibroblasts derived from an MSS patient. Cells were cultured as described in 6.1.2 Methods section 6.1.2 [21, 41].

6.2.2 Transcriptomic Analysis

Total RNA was extracted from control and patient fibroblasts in triplicate experiments using the miRNeasy micro kit (Qiagen, 217004) following the manufacturer's protocol. Transcriptomic analysis was carried out by next-generation sequencing (NGS) RNA sequencing at BMR Genomics Padova, Italy. RNA integrity was determined by analysis of extracted total RNA using a 2100 Bioanalyzer (Agilent Technologies) with RNA 6000 NanoChip. RNA concentrations were measured using Qubit RNA Assay Kit.

Libraries were prepared from total RNA using the Lexogen QuantSeq 3' mRNAseq Fwd kit, and their quality was evaluated by size analysis on a 2100 Bioanalyzer (Chip DNA HS) and quantified using the Qubit DNA HS assay kit (Thermo Fisher). Sequencing was carried out on an Illumina Nextseq 500, generating approximately 5 million/read per sample, SE75 format. Read preprocessing involved using fastp v0.20.0 [92] with specific parameters to remove adapter sequences and retain high-quality data. Passing filter reads were mapped to the genome reference (Homo sapiens) v105 of Ensembl repository using STAR v2.7.0 [93]. Alignments were elaborated by RSEM v1.3.3 [94] and sample-specific gene-level abundances were merged into a single raw expression matrix applying a dedicated RSEM command (rsem-generate-data-matrix). Genes with at least 10 counts in 3 samples were selected for further analysis. Differential gene expression was computed by edgeR [95] from raw counts in each comparison. A multiple testing correction procedure was applied, and genes with a false discovery rate (FDR) ≤ 0.1 and an absolute log fold change (FC) $> |0.5|$ were considered differentially

expressed. The heatmap displaying the 50 most differentially expressed genes (based on logFC) was generated using the pheatmap R library with unbiased hierarchical clustering [41].

6.2.3 Computer Simulation

To assess the significance of the overlap between the 237 biological processes identified in our study and the 898 processes reported previously [21] we generated 898 uniformly distributed pseudo-random integers ranging from 1 to 27,597 (number of biological processes in the GO database) with the Matlab algorithm. These numbers were associated with the biological processes in the GO database. This process of random number generation, association with GO terms, and calculation of the overlap with our set of 237 biological processes was repeated 10,000 times. Finally, the mean overlap across these 10,000 iterations was calculated to determine the expected overlap by chance [41].

6.2.4 Quantitative Real-Time PCR (q-PCR)

Total RNA was extracted from fibroblasts using TRIzol reagent. RNA was then retrotranscribed using the kit (High-Capacity cDNA Reverse Transcription Kit #4368813 from Applied Biosystems) according to the manufacturer's instructions. Quantitative real-time PCR (q-RT-PCR) was performed using SYBR Green chemistry (Sensifast SYBR HiRox #BIO-92020 from Bioline), using the CFX C1000 instrument (BIORAD). The expression levels of selected genes were normalised to the mean of the housekeeping gene GAPDH ($\Delta\Delta C_t$ method). The PCR primer sequences are listed above in the table.

One-way ANOVAs were performed to compare ΔC_t values for each gene between the two groups, followed by Benjamini-Hochberg correction to adjust p-values for multiple comparisons [41].

Table Primer sequences used

Gene name	FW sequence 3'-5'	REV sequence 3'-5'
ACAN	CTG CTA TGG AGA CAA GGA TGA G	CTG GAG ATG TTG CAT AAA AGA CC
ALDH1A1	AGC AGG AGT GTT TAC CAA AGA	CCC AGT TCT CTT CCA TTT CCA G
CTHRC1	CAA TGG CAT TCC GGG TAC AC	GTA CAC TCC GCA ATT TTC CCA A
CTTNBP2	CCG TGG GAC TTT ATG AGG AAG	GAG GGA TCA TGG AGG CAT AAG
ELAPOR2	CCT GGC AAC ATG AAA ACT TCC	TCA TTG TCA GAA CCT CCA GC
EXPH5	TGG GAC AGA AAG GAT GTG ATG	CTG GCT TTG GGA CAA ATG TAC
GAPDH	CTG GGC TAC ACT GAG CAC C	AAG TGG TCG TTG AGG GCA ATG
GPC4	AGG AAA CGG CAA TGA GGA TG	GAC GAA GGA TCA GTA TGT CTG G
LXN	ACA GAA CTA CAT CAA CTA CCA GC	GTG ATA CTT ATG TCC TCT TCC TGG
NRN1	ACA AGA CGA ACA TCA AGA CCG	TTA TCC CAC ATA TCT TTC GCC C
PCDH7	TGT CAA ACC AAT AAC AAG TAC AGC	ACT GCT TGG TGT TTC TGA CTC
PCOLCE2	GGG AAG TCA ACG ATG CTA GAA G	GTG ACC AAT AAA CCC ATC TGC
SIL1	GCT TCA CCT TCT GCC TCA GTC A	AAC ACC TCC AGG ACT TCG GCA T
VCAN	GGT GGT CTA CTT GGG GTG AG	GTG ATG CAG TTT CTG CGA GGA

6.2.5 Collagen Degradation/Internalisation Assay

Control and patient fibroblasts were seeded in 24-well plates (3×10^4 cells/well) or into ibidi μ -slide 8-well glass-bottom plates ($19,5 \times 10^2$ cell/well) for widefield or confocal analysis, under standard growth conditions. After 24 h, the growth medium was replaced with serum-free Dulbecco's Modified Eagle Medium (DMEM) and after 16 h cells were incubated with 10 μ g/mL of dye-quenched (DQ)- collagen (Invitrogen, Carlsbad, CA) for up to 5 h. Collagen degradation was monitored by detecting the green fluorescent signal using a fluorescence microscope (EVOS®FL, AMG, USA). The corrected total cell fluorescence (CTCF) was calculated for each cell using the formula: CTCF = integrated density – (area of selected cell $\times$ mean fluorescence of background readings) [96]. For confocal microscopy, cells were treated as described above and then exposed to LysoTracker (ThermoFisher) following the manufacturer's instructions. Images were acquired using a Zeiss LSM880 confocal microscope (Zeiss) using 495 and 647-nm lasers, as previously described [97]. Quantification of DQ-collagen internalisation in confocal images was performed using the CTCF calculation method described for the fluorescence microscope [41].

6.2.6 Collagenase Assays

Cell-free supernatants were tested for collagenase activity with the EnzChek Gelatinase/Collagenase assay kit (Invitrogen, Carlsbad, CA). Control and patient fibroblasts were seeded in 24-well plates 3×10^4 cells/well under standard growth conditions, and after 24 h medium was replaced with serum-free DMEM. Following a 16-hour incubation, cell supernatants were collected, centrifuged at 3000 g for 10 minutes at room temperature, and filtered through a 0.2 μ m Minisart sterile filter (Sartorius). Reaction mixtures were prepared in black optical bottom 96-well plates (Greiner BioOne, Monroe, NC) by combining 100 μ L of conditioned media with 20 μ L of the substrate (100 μ g/mL) and 80 μ L of reaction buffer. Plates were incubated at 37 °C under atmospheric conditions in a Synergy H1 microplate reader (BioTek, Winooski, VT). Kinetic fluorescence reads were measured after 7 s shaking every 5 min at 485 nm excitation/527 nm emission. Normalisation of collagenase activity was not required as the total protein amount was equivalent between control and patient fibroblast supernatants at the time of the experiment [41].

6.2.7 Cell Adhesion Assay

Control and patient fibroblasts were seeded at a density of 5×10^2 cells/well in 96-well plates and allowed to settle for 15 minutes at room temperature. The plates were then placed into an Incucyte® Live Cell Analysis System (Incucyte® S3/-Sartorius) maintained at 37 °C in a humidified CO₂ incubator. Four phase-contrast images of each well were acquired every 3 h for a total of 24 h using 10X objective. The resulting images were analysed using the Incucyte® Cell-by-Cell Analysis Software, and the exported data were further processed using GraphPad Prism 9 for statistical analysis. Statistical differences in the average phase object area between control and patient fibroblasts over time were determined using repeated measures ANOVA over the 9 time points. Fisher LSD post-hoc test was used to compare average phase object area values at the same time point between groups. A *p*-value <0.05 was the threshold for statistical significance.

6.2.8 Motility Assay

Cell motility was evaluated using the Incucyte Scratch Wound Assay protocol. Control and patient fibroblasts were seeded in 96-well ImageLock™ plates to achieve 100% confluency (2×10^4 cells/well) and incubated overnight under standard growth conditions. The following day, the 96-pin Incucyte WoundMaker (Sartorius) was used to create a precise and uniform wound simultaneously in each well. Cells were then washed twice with PBS and placed into the Incucyte® Live Cell Analysis System (Incucyte® S3/ -Sartorius). Two-phase contrast images from each well were acquired every 3 h for 24 h using 10X objective. The relative wound density was analysed using Incucyte® Scratch Wound Analysis Software Module, and the resulting data were exported for statistical analysis using GraphPad Prism.

Differences in the relative wound density between control and patient fibroblasts over time were determined using repeated measures ANOVA, as previously described for the average phase object area. A *p*-value less than 0.05 was considered statistically significant [41].

6.2.9 Transmission Electron Microscopy (TEM) of soluble ECM

Controls and patient fibroblasts, grown to 80% confluency, were incubated overnight in serum- and antibiotic-free medium. The following day, the conditioned medium was collected and centrifuged at 20,000 rpm to remove any residual cells or cellular debris. The protein

concentration of the conditioned medium quantified was on average 14.0 ± 1.8 and 10.8 ± 1.0 $\mu\text{g/mL}$ for control and patient fibroblasts, respectively.

The overall morphology of the collected ECM samples was characterised by transmission electron microscopy (TEM) using a CM 100 transmission electron microscope (Philips) equipped with a tungsten filament and operating at 100 kV. Specimens were prepared by negative staining, following a previously described protocol for protein molecules [98]. Briefly, a 5 μL drop of each ECM sample (concentration 10–15 $\mu\text{g/mL}$) was applied to a 200-mesh carbon-coated grid (Agar Scientific Ltd) and allowed to adsorb for 1 minute before applying a 100 μL drop of commercial Nano-W stain (2% organotungsten compound, Nanoprobes Inc.). Thereafter, the excess of stain was removed by touching the grid side with Whatman filter paper (Merck KGaA) and the grid was air-dried before TEM imaging. When necessary, the acquired micrographs have been post-processed using a high-pass or fast Fourier transform (FFT) bandpass filter by means of ImageJ v1.54 to reduce edge artefacts derived from low or over-exposure to the electron beam [41].

6.2.10 Mice

Woozy mice (CXB5/By-Sil1wz/J) purchased from The Jackson Laboratory (Stock No. 003777), housed at controlled humidity and temperature, were provided with a standard diet and water ad libitum. Our mouse colony was established through heterozygous breeding, and genotyping was performed using standard PCR methods. All procedures involving the mice were performed according to the protocol approved by the Institutional Animal Care and Use Committee of the Italian Ministry of Health (authorisation n° 596/2021-PR, issued pursuant to art. 31 of Legislative Decree 26/2014). Our animal housing facilities meet international standards and are regularly inspected by a certified veterinarian [41].

6.2.11 Histology and TEM of Woozy Mouse Achilles Tendon

Samples were carefully dissected from 7-month-old woozy ($n=3$) and control ($n=3$) mice, pinned on Sylgard dishes and fixed at room temperature with 3.5% glutaraldehyde in 0.1 M NaCaCO buffer (pH 7.2). The fixed samples were then stored at $+4^\circ\text{C}$ in the same fixative solution until further processing. For embedding, the samples underwent postfixation in a 2% OsO_4 solution in the same buffer for 2 hours, followed by block staining with uranyl acetate replacement. After dehydration, the specimens were embedded in Epon 812 epoxy resin (Electron Microscopy Sciences, Hatfield, PA, USA), following established procedures [99].

For histological examination by light microscopy, semithin sections (~700 nm) were cut using a Leica Ultracut R microtome (Leica Microsystem, Vienna, Austria) with a diamond knife (Diatome, Biel, Switzerland). These sections were stained for 3 minutes on a hot plate (55–60 °C) with a solution containing 1% toluidine blueO and 1% sodium borate (tetra) in distilled water. After washing and drying, sections were mounted with DPX media for histology (Sigma–Aldrich, Milan, Italy) and observed with a Leica DMLB light microscope connected to a DFC450 camera equipped with Application Suite v 4.13.0 for Windows (Leica Microsystem, CMS GmbH, Switzerland).

For TEM, ultrathin sections (~50 nm) were cut using the same Leica Ultracut R microtome and a Diatome diamond knife. These sections were double-stained with uranyl acetate replacement and lead citrate. Imaging was performed using a 120 kV JEM- 1400 Flash Transmission Electron Microscope (Jeol Ltd, Tokyo, Japan) equipped with CMOS camera Matataki and SightX Viewer software (Jeol Ltd, Tokyo, Japan) at 80 kV [41].

6.3 Methods of Molecular Characterisation of A Newly Identified Pathogenic Variant in The SIL1 Gene Causing Marinesco-Sjögren Syndrome

6.3.1 Patient Information

The proband, a 4-year-old female, was referred for a neurodevelopmental delay at the age of 2 to the Laboratory of Medical Genetics at St. Sophia's Children's Hospital, Athens, Greece. She is the first child of non-consanguineous Greek parents. Her father has two children from previous relationships, a 19-year-old healthy male and a 23-year-old healthy female. The proband also has an 18-year-old maternal half-sister.

This genetic study received ethical approval from the St. Sophia Children's Hospital Scientific and Ethics Committee (Approval number: 3669/12-02-18). All patient data were processed and stored according to the guidelines of the General Data Protection Regulation (GDPR). Written informed consent was provided by the patient's parents.

6.3.2 Whole-Exome Sequencing and Targeted Sanger Sequencing Analyses

Proband-only WES (~20,000 genes) was performed, using genomic DNA extracted from whole

blood. Library preparation was carried out using IDT xGen Exome Research v2 kit (Integrated DNA Technologies). The resulting libraries were subjected to paired-end sequencing on an Illumina NextSeq 500 platform. Bioinformatic analysis of the WES data yielded 61,849,178 reads and 25,446 variants. The percentage of regions with at least 20X coverage was 98% and the mean coverage was 83X. Variant analysis was conducted using VarSome Clinical platform (Saphetor SA) (<https://varsome.com/>) and was based on the phenotype-driven strategy [100]. Variant classification was based on American College of Medical Genetics and Genomics (ACMG) guidelines [101].

Targeted Sanger sequencing for the detected variant was performed in the proband and her parents. Polymerase Chain Reaction (PCR) for amplification of exon 9 of the *SIL1* gene was performed using specific primers designed with NCBI Primer Blast (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>).

The primer sequences flanking exon 9 of the *SIL1* were:

5'-GGGTATAGCTGTAAGTGAAGTG-3' (forward);

5'- CTCACATGACATACTCCCTGAC-3' (reverse).

PCR reactions were performed using Hot Start Taq (Qiagen, Hilden, Germany) polymerase and both strands were sequenced using the Big Dye® Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific SA, Waltham, Massachusetts, USA). Capillary electrophoresis was performed in ABI 3500 Genetic Analyzer sequencing system (Thermo Fisher Scientific SA, Waltham, Massachusetts, USA).

6.3.3 Preparation of Recombinant *Sil1*

To express *Sil1* in COS7 cells, the ORF of the human *SIL1* gene, its variant c.1024G>A (p.E342K) and the pathogenic missense variant c.1370T>C (p.L457P) were cloned into the mammalian gene expression vector pRP-CMV (VectorBuilder Inc.).

To express *Sil1* in *Escherichia coli* (*E. Coli*), the *SIL1* WT and *SIL1* p.E432K variant, both lacking the first 31 amino acids at the N-terminus, were cloned into the pGEX6P1 vector (GenScript), enabling the recombinant proteins to have an N-terminal GST tag.

BL21(DE3) competent cells (Novagen) were transformed with 100 ng of plasmid, and colonies were selected by growth at 37°C on Luria-Bertani (LB) agar plates containing ampicillin 50 µg/ml. Transformed bacteria were suspended in LB Miller broth until the absorbance at 600 nm reached 0.5–0.6. Expression of the recombinant proteins was induced by isopropyl-β-D-

thiogalactopyranoside 0.5 mM for 3 hours at 25°C. After centrifugation at $4,000 \times g$ for 15 minutes at 4°C, the bacterial pellets were frozen at -80°C until use.

For protein purification, bacterial pellets were resuspended in a buffer containing HEPES 50 mM, NaCl 400 mM, pH 7.4, 10% glycerol, DNase I, and a cocktail of protease inhibitors. The cells were lysed by sonication on ice for 30 minutes (10 seconds on, 10 seconds off; amplitude 60%, cycle 0.5). The lysates were then centrifuged at $16,000 \times g$ for 1 hour at 4°C, and the supernatants were loaded onto Glutathione Sepharose 4B agarose resin pre-equilibrated with HEPES 50 mM, NaCl 400 mM pH 7.4. The affinity chromatography purification was carried out at room temperature. The column was first washed in HEPES 50 mM, NaCl 500 mM pH 7.4, with the addition of ATP 5 mM and $MgCl_2$ 15 mM to remove potential contamination from bacterial chaperones. The resin was incubated overnight with HRV-3C protease to cleave the GST-tag and the recombinant Sil1WT, Sil1 p.E432K were eluted in HEPES 50mM, NaCl 400 mM pH 7.4. All the eluted fractions were analysed by 10% SDS-PAGE under reducing conditions and stained overnight with ReadyBlue Protein Gel Stain.

The purified proteins were then concentrated with Amicon devices (MWCO 10 kDa) and the protein concentration was estimated by measuring the absorbance at 280 nm using $\epsilon=33.015$ deduced by ProtParam [102].

6.3.4 Cell Culture Conditions

Fibroblasts obtained from the patient carrying the p.E342K variant were cultivated as described in the 6.1.2 Methods section, along with primary human dermal fibroblasts, used as the control cell line for patient fibroblasts, and the other patient-derived cells.

6.3.5 Cell Transfection, Treatments and Western Blotting

For COS-7 cell transfection, 250,000 cells/well were seeded in 6-well plates containing a coverslip in each well. The following day, cells were transfected with 3 μ g of respectively SIL1 WT, SIL1 p.E342K and SIL1 p.L457P plasmids, using Lipofectamine 3000 (Invitrogen #L3000015) according to manufacturer's instructions.

For immunofluorescence analysis, after 18 hours post-transfection, cells were treated for 2 hours with cycloheximide (25 μ g/ml) and fixed. For Western blot analysis, transfection was stopped after 72 hours, and cells were lysed and processed as described in our previously published studies [21, 32, 41], with the exception of the use of precast gel (Genscript #M00659

SurePAGE™, Bis-Tris, 10x8, 8-16%, 12 wells).

The following primary antibodies were used for WB as indicated: SIL1 (Sigma Prestige #S-HPA011949), CKB (Abcam #ab92452), CTHRC1 (Abcam #ab5739), FHL1 (Proteintech #10991-1-AP), KCTD12 (Proteintech #15523-1-AP), PDLIM5 (Proteintech 10530-1-AP), Ubiquitin (Calbiochem #662099), GAPDH (Santacruz #sc-32233).

MG132 proteasome inhibition was performed by seeding the cells at 70% of confluency in 60-mm or in 24-well dishes containing coverslips. The following day, cells were treated with MG132 20µM (Calbiochem #474790) for 6h. Finally, the cells have been processed for Western blotting and immunofluorescence confocal microscopy as appropriate.

6.3.6 *Fluorescence Immunostaining and Confocal Microscopy*

Cells seeded on coverslips were fixed in ice-cold methanol at -20°C for 5 minutes once they reached 70% confluence. Cells were then processed according to our previously published studies [21, 32]. Primary antibodies used in IF are SIL1 (Sigma Prestige #S-HPA011949) and Lamp2a (Santacruz #sc-18822). Fluorescence detection was performed using an LSM800 Zeiss confocal microscope, and the resulting images were post-processed and analysed using Fiji software.

6.3.7 *Native-PAGE*

Cells were lysed under gentle conditions as described by Roychowdhury et al. [103]. The native lysis buffer consisted of Tris 20 mM (pH 7.4), KCl 20 mM, MgCl₂ 5 mM, and 0.01% NP40, supplemented with protease and phosphatase inhibitor cocktails (Merck). Cells suspended in the lysis buffer were incubated for 10 minutes on dry ice, followed by 2 minutes at 37°C in a water bath. This step was repeated three times, then the samples were incubated on ice for 30 minutes. After centrifugation for 20 minutes at 12,000 rpm at 4°C, both the supernatants and pellets were collected. Pellets were treated on ice for 30 minutes at 4°C with native lysis buffer added with Urea 8M, then sonicated and centrifuged for 20 minutes at 4°C (12,000 rpm). Protein concentrations in both fractions were determined using the Bradford assay.

Proteins solubilised in native buffers were analysed by native-PAGE, while proteins solubilised in urea buffer were analysed by SDS-PAGE. For native-PAGE electrophoresis, stacking gel (4% acrylamide), running gel (10% acrylamide) and running buffer (1X) were prepared without SDS, according to the indications of Sino Biological Inc (<http://www.assay->

protocol.com/molecular-biology/electrophoresis/native-page.html). Proteins loaded on native-PAGE were not heated, but simply diluted with Tris-HCl 62.5 mM pH6.8, 25% glycerol and 1% Bromophenol Blue. The electrophoresis run was performed at 100V with the Bio-Rad apparatus placed on ice.

6.3.8 RNA Extraction and Reverse Transcriptase Quantitative PCR

Total RNA was extracted from cells using QIAzol (RNeasy Lipid Kit, QIAGEN Inc., Valencia, CA), according to the manufacturer's instructions. RNA quality and concentration were determined by NanoDrop 2000 spectrophotometry (Thermo Scientific, Wilmington, DE, USA) and verified by 1% agarose gel electrophoresis. 1 µg of total RNA was reverse transcribed into cDNA using the High-Capacity cDNA Archive kit in accordance with the manufacturer's recommendations (Applied Biosystems).

Relative quantification of gene expression was performed by reverse transcriptase quantitative PCR (RT-qPCR) using the CFX96 Real-Time PCR Detection System (Bio-Rad) and the SensiFAST SYBR kit (Bioline). Primers used for amplification were designed using IDT PrimerQuest Tool software and synthesised by Integrated DNA Technologies (IDT, Coralville, IA, USA). The primer sequences are shown in Table in 6.2.4 Methods section.

Data analysis was performed using CFX Manager software (Bio-Rad). The relative expression of target genes was calculated using the $\Delta\Delta C_t$ method, normalising the expression of target genes to the reference gene (GAPDH) [104]. The results are shown as log fold change with respect to the control cells. Statistical analysis was performed using a Multiple unpaired t-test to compare ΔC_t values of HF-P2 to HF-CTRL.

6.3.9 Electron Microscopy Analysis

Approximately 2×10^6 fibroblast cells cultured in monolayers were prepared for EM as previously described [21, 32]. Cells were detached using trypsin, centrifuged at $180 \times g$ for 2.5 minutes, and washed three times with PBS at 37 °C. Fixation was performed with 3.5% glutaraldehyde in sodium cacodylate buffer (NaCaCO) 0.1 M for 1 hour, and the fixed cells were stored at 4 °C. For embedding, cells were post-fixed in 2% OsO₄ in the same buffer for 2 hours and block-stained with saturated uranyl acetate replacement. After dehydration, specimens were embedded in Epon 812 epoxy resin. Ultrathin sections were cut using a Leica Ultracut R microtome (Leica Microsystem, Austria) with a Diatome knife (DiatomeLtd., CH-

2501 Biel, Switzerland) and then double-stained with uranyl acetate replacement and lead citrate. Sections were examined and photographed using a JEM-1400Flash transmission electron microscope (Jeol Ltd., Tokyo, Japan) equipped with a Matataki digital camera and SightX Viewer software (ver. 2.1.26.1818, Jeol Ltd., Tokyo, Japan).

6.3.10 Label-Free Proteomics and DE Protein Data Analysis

Cell samples were lysed in buffer B Strong (UREA 6 M, Tris Base 100 mM, 2% CHAPS, 1% Triton X, DTT 50 mM), and processed by Filter Aided Sample Preparation (FASP) method. As described previously [21] protein concentration was evaluated through Bradford assay (Bio-Rad Laboratories., USA) to digest 30 µg of protein by trypsin (0,5 µg/µl Trypsin, Sigma-Aldrich, St. Louis, MI, USA). Tryptic peptides were analysed in triplicate with nanoLC-MS/MS using the UltiMate™ 3000 UPLC (Thermo Fisher Scientific, Waltham, MA, USA) chromatographic system coupled to the Orbitrap Fusion™ Tribrid™ (Thermo Fisher Scientific) mass spectrometer with the EASY-spray Acclaim™ PepMap™ C18 (75 µm ID, 15 cm L, 2 µm PS, Thermo Fisher Scientific) chromatographic column in a total run time of 65 min and a chromatographic gradient from 5% to 90% of acetonitrile. Label-free protein identification and quantification were as in Di Stefano et al. [105]. Raw data were processed using Thermo Proteome Discoverer (PD) version 2.4.0.305 (Thermo Fischer Scientific). In order to perform a robust quantification, we removed all contaminant proteins from the dataset and set the parameters “Abundance Ratio Variabilities [%]” less than 50.00 in the ratio and number of “Unique peptides” greater than one. The volcano plot was generated from the statistical analysis performed using PD software and highlighted significantly up- and down-regulated proteins, with the *p*-value indicating the statistical significance of the “Protein Abundance Ratio”. Similarly, the heatmap was generated using PD software, considering the individual protein abundance values for each quantified protein in each analytical replicate. Raw protein abundances were loaded and analysed using BigOmics Analytics: Omics Analysis Software (<https://bigomics.ch/>).

6.4 Methods of Assessment of The Woozy Mouse Model Phenotype

6.4.1 Animal models

A total of 44 mice, 22 *Sil1* homozygous mice (*Sil1^{wz}*) and 22 *SIL1* heterozygous mice (*Sil1^{ht}*), equally distributed in sex, were used for the characterisation of this model. *Sil1^{wz}* mice were produced by crossing *Sil1^{ht}* mice (CXB5/By-Sil1wz/J, JAX stock #003777), supplied by The Jackson Laboratory (Maine, USA). Mice were housed together with littermates and aged until the weaning dates (PND 23-24). The genotype of animals was confirmed using the standard PCR assay (Jackson Laboratories Protocol #22452) performed on DNA isolated from an ear punch. PCR products were analysed by 1.5% agarose gel electrophoresis, and genotypes were determined based on fragment size: *Sil1^{wz}* = 758bp; *Sil1^{ht}* = 626bp and 758bp; *Sil1^{wt}* = 626bp. Mice were housed in groups of four to five per cage (Tecniplast, Buguggiate (VA), Italy) with wood chips as bedding (Caipet, San Severo (FG), Italy), in a temperature-controlled room with a 12-hour light-dark cycle and free access to food and water. Starting at 5 weeks of age, a first cohort of animals was weighed weekly and tested on the accelerating rotarod and beam walking apparatus on two separate days. At 10 weeks, a nesting building test was performed the night before the first motor test. At 14 weeks, the nesting building test was repeated, followed by the pole test on two consecutive days. At 16 weeks, the second pole test and the inverted grid test were conducted. The day after the final test, half of the animals from each experimental group were randomly selected and sacrificed by cervical dislocation. The other animals were maintained in the same housing conditions until the 26th week. A second cohort of animals underwent testing in the novel object recognition and Y-maze tests at 5 and 14 weeks of age, on two consecutive days.

All experimental procedures have been approved by the CAST animal welfare survey board, on behalf of the Italian Ministry of Health (formal license 596/2021 PR, delivered to M.S.), and were conducted following European Community specifications regarding the care and use of laboratory animals.

6.4.2 Accelerating Rotarod Test

In order to evaluate sensorimotor deficits [106], the motor functions and neuromuscular coordination were assessed with the accelerating Rotarod 7650 model (Accelerating Model, Ugo Basile, Biological Research Apparatus, Varese, Italy), as previously described [107].

The day of the test, mice were trained for three times, for 60s at a constant acceleration of 4 rpm before starting. For the test, animals were placed on the rotating bar (constant at 7 rpm) and allowed to acclimatise for 30s. The acceleration began at 7 rpm and accelerated up to 40 rpm, at a constant rate of 0.11 rpm/s for a maximum of 300 s. Motor coordination and fatigue were evaluated by measuring the latency to fall from the bar, which served as the endpoint. Animals performed three times, with a rest of 30 min between each trial, and the average time was used for statistical analysis. Animals were weekly tested from their 5th to their 14th week of life. Instead of representing data as the individual latency time expressed in s, we choose to represent the values as the percentage (%) with respect to the individual maximum time in the apparatus.

6.4.3 *Beam walking test*

Mice were trained two days before being tested to walk along a homemade PVC round bar 0.8 cm wide, 80 cm long, suspended 40 cm above bedding material, with a safe platform at the end [52]. On the day of the test, animals were given three trials, waiting at least 5 min between each trial. Mice were video recorded during their performance and the time to traverse the beam, the number of hindfoot missteps, and eventual falls during the trials were scored. The average time to travel the beam and reach the platform, together with the number of missteps (considered when the mice slid with their hind limbs along the round beam), scored during the three trials were used for statistical analysis. The video analysis was performed by an operator blinded to the experimental conditions. A maximum time of 60 seconds was recorded if an animal fell or took longer to cross the beam. Mice were tested weekly from 5 to 12 weeks of age.

6.4.4 *Pole Test*

A homemade vertical PVC round pole 0.8 cm wide, 50 cm long, covered with a thin rope twisted on its surface, was positioned on a base and placed into the mouse home cage, as previously described [108]. During the morning of the test, mice were trained to turn themselves from upward on top of the pole and descend the length of the pole to return to their home cage. On the day of testing, animals were given three trials and were video recorded. The time required for the animals to orient themselves facing in a downward direction (T-turn) and to descend the base of the pole (T-total) were scored. The average T-turn and T-total times were used for statistical analysis. A cutoff of 30 s was chosen as the maximum T-turn time, while a

cutoff of 60 s was chosen as the maximum T-total time. Animals were tested at their 14th and 16th week of life.

6.4.5 *Inverted Grid Test*

The inverted grid test was used to assess grip strength and muscle endurance [109]. Mice were placed onto a suspended stainless-steel wire grid (40 cm x 40 cm, wires spaced 1 cm apart) located 40 cm above the floor over a soft paper chip bedding. After a mouse was placed at the centre of the wire grid, the apparatus was inverted, and the test began. The trial was videotaped, and latency to drop down from the wire grid (cutoff time 120 s), together with immobility time, time spent in the different zones of the apparatus, and the number of times the animals left the grid with their forelimbs or hindlimbs were scored and considered for the analysis. An operator blinded to the experimental conditions evaluated and scored all the parameters. Mice were tested two days after the last motor test at their 16th week of life.

6.4.6 *Nesting Building*

Approximately 1h before the dark phase, mice were individually moved to a testing cage equipped with wood chip bedding and a 3 g piece of paper folded twice and placed in the back part of the cage. The following morning, an operator removed the cage lid and took a picture of the entire cage, and of the opened rectangular piece of paper from above, to evaluate the movement of the paper inside the cage, the degree of fold opening, and the percentage of gnawing by the mouse, respectively. Mice were individually caged for their entire dark phase, from 6 p.m. to 9 a.m., at their 10th and 14th week of life.

Similarly to previous studies [81, 110], we used the following scoring to evaluate the individual parameter: paper movement on a scale of 1 to 5 (1 = no movement; 3 = paper moved to the middle of the cage; 5 = paper moved to the end of the cage); degree of opening on a scale of 1 to 3 (1 = paper closed; 2 = paper half opened; 3 = paper completely opened); gnawing percentage on a scale of 1 to 5 (1 = not gnawed paper at all; 3 = 20% of paper gnawed; 5 = more than 40% of paper gnawed). Three operators blinded to the experimental conditions evaluated and scored the test parameters, and the average of each parameter was used for statistical analysis.

6.4.7 Novel object recognition test

The NOR was performed as previously described [111]. Briefly, two plexiglass (30 x 15 cm) cages were used for the experiment. During the habituation phase mice were individually placed 10 min per day for 2 consecutive days in the empty cages. On day 3, mice were placed in the cage and exposed to two identical plastic objects 15 cm apart and allowed to explore the objects for 8 min before returning to their home cage. Between each trial, objects were thoroughly cleaned with 70% ethanol and dried to remove any scent residue. On day 4, mice were returned to the experimental cage where one of the two familiar objects was replaced with a differently shaped object (novel object). A 5 min-probe test was recorded while mice were allowed to freely explore the familiar and novel objects. NOR performance scores were quantified by monitoring the time spent exploring the familiar and the novel objects. Mice behaviour was considered explorative when the animal heads leaned within 1 cm from the object and vibrissae moved. Proximity, chewing, or standing near the object without active investigation were not considered exploratory behaviour.

The parameters included in the analysis were: the percentage of time spent with the novel object and the discrimination index (DI), calculated as $[(A - B) / (A + B)] \times 100$, where A is the time spent exploring the novel object and B is the time spent exploring the familiar object.

6.4.8 Y-Maze

Working memory and exploratory activity were measured by employing a Y-maze apparatus as described elsewhere [112]. The apparatus consists of a Y-shaped structure made of black plexiglass walls with dimensions 39.5 x 8.5 x 13 cm. Mice, which had no prior experience with the maze, were individually placed at the end of one arm of the apparatus and allowed to move freely through the maze. Each 10-minute session was recorded and the number of arm entries and alteration patterns analysed. The percentage of alternations, a measure of spatial working memory, was calculated using the formula: % Alternations = (Total Number of Alternations / Number of Arm Entries) $\times$ 100.

6.4.9 Molecular Analysis

A total of 3 *Sil1^{wz}* and 3 *Sil1^{ht}*, equally distributed in sex, were randomly selected for the molecular analysis. Two days after the last motor assessment test, mice were sacrificed by

cervical dislocation and tissues were quickly frozen and stored at -80°C until molecular assays. Frozen mouse gastrocnemius and soleus muscles ($\sim 100\text{mg}$) were homogenized in ice-cold lysis buffer (Tris-HCl 0.5 M [pH 7.4], NaCl 1.5 M, 2.5% deoxycholic acid, 10% NP-40, EDTA 10 mM) using ultrasound (10x pulse, 0.5 amplitude, 50 efficiency). The concentration of the protein supernatant was determined using the Bradford assay. The proteins were mixed with Laemmli buffer (Tris-HCl 31.5 mM [pH 6.8], 10% glycerol, 1% SDS, 0.005% Bromophenol Blue), denatured for 8 min at 95°C , and the same amount of protein ($25\text{ }\mu\text{g}$ per well) was separated by SDS-PAGE. After electrophoresis, the separated proteins were transferred onto a nitrocellulose membrane using a wet/tank blotting system (Bio-Rad, Hercules, CA, USA). Consequently, the membrane was blocked using TBS-T (TBS + 1% Tween 20) + 5% milk/BSA (based on the Ab used) for 1 h at RT and was then incubated overnight at 4°C with antibodies specific for BiP (BD Bioscience #610979), Pdi (Abcam #ab2792), pEIF2 α (Cell Signaling #9721), Chop (SantaCruz #Sc-7351), Rab11 (BD Bioscience #610657), LC3 (Novus Biologicals – Biotechnne #NB100-2220), and Ckb (Abcam #ab92452). After primary antibody incubation, the membrane was washed three times with TBS-T (0.1%) and incubated with H&L chain specific peroxidase-conjugate secondary anti-rabbit (Calbiochem #401315) or anti-mouse (Merck Millipore #401215) antibodies for 1 h at RT before a final three washes with TBS-T. Antibody binding was detected using the Alliance Atom (UVITEC, Cambridge, UK). GAPDH protein (SantaCruz #Sc-32233) abundance was used as reference control. The data are expressed relative to GAPDH expression.

6.4.10 Statistical Analysis

All data are expressed as the mean $\pm$ SEM, and the statistical analysis was performed with GraphPad Prism 10 software. The multiple t-test, corrected with the Holm-Sidak method, was used to compare data obtained from the locomotor tests performed between the groups. Unpaired Mann–Whitney t-test was used to analyse the individual parameters not dependent/influenced by other parameters of the same test (e.g. the total latency and the immobility time of the inverted grid test). Spearman’s correlation analysis was used to measure the strength and direction of the relationship between the individual parameters observed in the motor and cognitive tests. P value < 0.05 was set as the threshold for statistical significance.

7. Bibliography

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