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Muscleblind-like1 undergoes ectopic relocation in the nuclei of skeletal muscles in myotonic dystrophy and sarcopenia

M. Malatesta,[1] M. Giagnacovo,[2]
M. Costanzo,[1] B. Cisterna,[1] R. Cardani,[3]
G. Meola[3,4]

[1]Dipartimento di Scienze Neurologiche, Neuropsicologiche, Morfologiche e Motorie, Sezione di Anatomia e Istologia, Università di Verona, Italy; [2]Dipartimento di Biologia e Biotecnologie "Lazzaro Spallanzani", Laboratorio di Biologia cellulare e Neurobiologia, Università di Pavia, Italy; [3]Laboratorio di Istopatologia muscolare e Biologia molecolare, IRCCS Policlinico San Donato, Milano, Italy; [4]Dipartimento di Neurologia, IRCCS Policlinico San Donato, Università di Milano, Italy

Abstract

Muscleblind-like 1 (MBNL1) is an alternative splicing factor involved in postnatal development of skeletal muscles and heart in humans and mice, and its deregulation is known to be pivotal in the onset and development of myotonic dystrophy (DM). In fact, in DM patients this protein is ectopically sequestered into intranuclear *foci*, thus compromising the regulation of the alternative splicing of several genes. However, despite the numerous biochemical and molecular studies, scarce attention has been paid to the intranuclear location of MBNL1 outside the *foci*, although previous data demonstrated that in DM patients various splicing and cleavage factors undergo an abnormal intranuclear distribution suggestive of impaired RNA processing. Interestingly, these nuclear alterations strongly remind those observed in sarcopenia *i.e.*, the loss of muscle mass and function which physiologically occurs during ageing. On this basis, in the present investigation the ultrastructural localization of MBNL1 was analyzed in the myonuclei of skeletal muscles from healthy and DM patients as well as from adult and old (sarcopenic) mice, in the attempt to elucidate possible changes in its distribution and amount. Our data demonstrate that in both dystrophic and sarcopenic muscles MBNL1 undergoes intranuclear relocation, accumulating in its usual functional sites but also ectopically moving to domains which are usually devoid of this protein in healthy adults. This accumulation/delocalization could contribute

to hamper the functionality of the whole splicing machinery, leading to a lower nuclear metabolic activity and, consequently, to a less efficient protein synthesis. Moreover, the similar nuclear alterations found in DM and sarcopenia may account for the similar muscle tissue features (myofibre atrophy, fibre size variability and centrally located nuclei), and, in general, for the aging-reminiscent phenotype observed in DM patients.

Introduction

The Muscleblind-like (MBNL) family of proteins is a class of tissue-specific regulators of developmentally programmed alternative splicing;[1-3] a few studies have also proposed a role for these proteins in translational control through a modulation of RNA stability.[4,5] In eukaryotic cells, gene primary transcripts (pre-mRNAs) undergo extensive modifications before generating mature mRNA to be exported to the cytoplasm, and splicing represents a key co- and post-transcriptional step. In Vertebrates, most of the pre-mRNAs are alternatively spliced, allowing the synthesis of different protein isoforms from the transcripts of a single gene.[6] Defects in alternative splicing processes can contribute to pathogenesis, as demonstrated for a growing number of genetic diseases, including myotonic dystrophies.[7]

Myotonic dystrophies (DMs) are autosomal dominant disorders characterised by a variety of multisystemic features among which myofibre dystrophy with increased number of centrally located or clumped nuclei,[8] myotonia (muscle hyperexcitability), dilated cardiomyopathy, defects in cardiac conduction,[9] insulin-resistance, cataracts,[10] and disease-specific serological abnormalities.[11-13] Two forms of DM have been described: DM1-Steinert's disease (OMIM 160900), caused by an expanded (CTG)n nucleotide sequence in the 3' untranslated region of the Dystrophia Myotonic Protein Kinase (DMPK) gene (OMIM 605377) on chromosome 19q13;[14-16] and DM2 (OMIM 602688), caused by the expansion of the tetranucleotidic repeat (CCTG)n in the first intron of the Zinc Finger Protein (ZNF9 gene, now called CNBP) (OMIM 116955)[17,18] on chromosome 3q21.[19]

Both CUG- and CCUG-containing transcripts are retained in the cell nucleus and accumulate in RNA- and protein-containing aggregates called *foci*[18] where MBNL1, 2 and 3 proteins can be sequestered,[20] thus compromising the regulation of alternative splicing.[21] In particular, MBNL1 (which directly binds both CUG and CCUG RNA repeats,[22,23] is involved in the postnatal development of skeletal muscles and heart in humans and mice.[24-26]

In the pathogenesis of DMs, aberrant regulation of the alternative splicing events has been found for more than 30 genes.[27] Importantly, MBNL1 directly regulates the alternative splicing of the genes coding for cardiac troponin T (cTNT),[1,28] myomesin 1 (MYOM1),[29] non muscle myosin heavy-chain (MYH14),[30] bridging integrator-1 (BIN1, required for the biogenesis of muscle T tubules),[31] skeletal muscle chloride channel (CLCN1),[11,32] Ca(V)1.1 (a calcium channel that controls skeletal muscle excitation-contraction coupling),[33] and insulin receptor (IR).[1,34] These misregulated post-transcriptional processes may account for several of the multiple symptoms observed in DM patients, thus making MBNL1 one of the best examples of regulatory splicing factors involved in the onset and development of the disease symptoms. However, despite the numerous biochemical and molecular studies on the splicing activity of MBNL1 and its sequestration in the DM *foci*, scarce attention has so far been paid to its intranuclear distribution pattern outside the *foci*.[35] It has been recently found that the ribonucleoprotein (RNP)-containing structures, *i.e.* perichromatin fibrils (PF), perichromatin granules (PG) and interchromatin granules (IG)[36] as well as the molecular factors responsible for pre-mRNA transcription and maturation undergo massive rearrangement

in the nuclei of skeletal muscles from DM1 and DM2 patients:[37] the precise intranuclear location of these RNP constituents is an essential prerequisite for pre-mRNA synthesis and processing to correctly take place, and they are considered as highly sensitive markers of nuclear activity.[38] Interestingly, the abnormal RNP distribution in DM muscle cells suggests an impairment of pre-mRNA processing[37] and strongly reminds the nuclear alterations typical of sarcopenia[39-41] *i.e.*, the loss of muscle mass and function which physiologically occur during ageing[42] and is characterised by myofibre atrophy, fibre size variability and centrally located nuclei.[43] This evidence allowed to hypothesize that common nuclear mechanisms might be responsible for skeletal muscle wasting in sarcopenia and in different muscular pathologies.[44-46]

On the bases of this rationale, in the present work the nuclear localization of the key alternative splicing factor MBNL1 was analyzed in skeletal muscles from healthy subjects and myotonic dystrophy type 1 and 2 patients as well as from adult and old (sarcopenic) mice; immunoelectron microscopy and biochemistry were used in the attempt to elucidate possible changes in the distribution and amount of MBNL1.

Materials and Methods

Skeletal muscle sample processing

The biopsies were taken, under sterile conditions, from the *biceps brachii* muscles of adult patients affected by DM1 (three subjects aged 18-46) or DM2 (three subjects aged 53-60) as well as of three healthy donors (aged 18-36), after informed consent; the procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation of the IRCCS Policlinico San Donato. The patients affected by DM2 were the oldest ones, due to the late onset of the disease symptoms, while the patients affected by DM1 are characterised by an earlier onset of muscle dystrophy; however, all the subjects were in the adulthood range, thus excluding possible age-related changes in nuclear features. The histological diagnosis was performed on serial sections processed for routine histological or histochemical staining, based on the clinical criteria set by the International Consortium for Myotonic Dystrophies.[47] Immediately after removal, the biopsies were cut into small fragments and fixed by immersion in 4% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, for 2 h at 4°C. Some other muscle samples were frozen in cooled isopentane

and preserved in liquid nitrogen.

Six adult (9 months of age) and six old (28 months of age) male Balb-c mice were bred under controlled environmental conditions with a 12 h light/dark cycle, and fed *ad libitum* on a standard commercial chow. The experimental protocols comply with the guidelines of the Italian Ministry of Health as well as with internationally recognized guidelines. The mice were deeply anaesthetised with pentobarbital (50 mg/Kg i.p.). Six mice (three per age group) were then perfused *via* the ascending aorta with a brief prewash of 0.09% NaCl solution followed by 300 mL of a fixative solution containing 4% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4 at 4°C. *Quadriceps femoris* muscles were quickly removed and placed in the same fixative solution for 2 h at 4°C. This muscle was chosen because it is mainly composed by fast type II fibers, that are prone to sarcopenia.[48] After fixation, all muscle samples were washed in phosphate buffer saline (PBS), deepen in 0.5 M NH_4Cl in PBS for 45 min to block free aldehydes, dehydrated with ethanol and embedded in LR White resin. Ultrathin sections were collected on Formvar-carbon coated nickel grids and used for the immunocytochemical analyses. In addition, for biochemical analyses six mice (three per age group) were killed after ether anaesthesia, and the *quadriceps femoris* muscles were dissected, immediately frozen in cooled isopentane and preserved in liquid nitrogen.

Immunogold labelling

Sections were floated for 3 min on normal goat serum diluted 1:100 in PBS and then incubated for 17 h at 4°C with a rabbit polyclonal anti-MBNL1 antibody (kind gift of Prof. C.A. Thornton[24]) diluted 1:50 with PBS containing 0.1% bovine serum albumin (Fluka) and 0.05% Tween 20. After rinsing, sections were floated on normal goat serum, and then allowed to react for 30 min at room temperature with a 12 nm gold-conjugated goat anti-rabbit secondary antibody (Jackson ImmunoResearch Lab., Inc., West Grove, PA, USA) diluted 1:10 in PBS. Finally, the sections were rinsed and air-dried. As controls, some grids were incubated without the primary antibody and then processed as described above. To reduce chromatin contrast and selectively reveal nuclear RNP constituents, the sections were treated according to the EDTA method,[49] and then observed in a Philips Morgagni TEM operating at 80kV and equipped with a Megaview II camera for digital image acquisition.

Quantitative assessment of the immunolabelling was carried out by estimating the gold particle density over different nuclear domains in samples treated in the same run. Based on the widely accepted nuclear nomenclature,[36]

we considered the two major compartments, *i.e.* the nucleolus and the nucleoplasm (consisting of chromatin areas and the interchromatin space); it should be underlined that in DM samples the *foci* were excluded from the nucleoplasmic area and measured separately. In addition, we considered as nuclear domains the IG clusters and, in order to get an estimate of the surface covered by PF (that is hardly assessable by direct morphometry), we defined a domain called *interchromatin space minus IG*, which was obtained by subtracting the condensed chromatin plus IG areas from the whole nucleoplasmic area.[50,51] The surface areas of the different domains were measured in fifteen randomly selected electron micrographs (x22,000) of myonuclei from each patient or mouse using a computerized image analysis system (AnalySIS Image processing, Soft Imaging System GmbH, Muenster, Germany). Background evaluation was carried out on resin (in the areas devoid of tissue) of immunolabelled samples as well as on the tissue of control samples. Gold particles present over the domains were counted, and the labelling density was expressed as the number of gold particles/μm^2. For each analyzed variable, the Kolmogorov-Smirnov two-sample test was performed in order to verify the hypothesis of identical distributions among animals of each group. The data for each variable were then pooled according to the experimental groups, and the mean ± standard error of the mean (SE) was calculated. Statistical analysis of the results was performed by the one-way ANOVA test. Statistical significance was set at $P \leq 0.05$.

Western blotting analysis

For protein extraction, skeletal muscles were deeped for 20 min in ice in cold lysis buffer (1 mL for 100 mg tissue) containing 50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% Nonidet P40, 0.25% Na-deoxycolate, 0.1% Sodium Dodecyl Sulfate (SDS), protease inhibitor cocktail (Sigma-Aldrich), in distiller water. Then, the samples were homogenised and centrifuged at 13,000 rpm for 15 min at 4°C, the supernatants were collected, and proteins were quantified by the Bradford method. Twenty μg of proteins per sample were loaded and separated by electrophoresis on 12% SDS-polyacrylamide gels, and transferred onto nitrocellulose membranes (Amersham Biosciences, Inc., Piscataway, NJ, USA). Non-specific bindings were blocked by incubation in 5% non-fat dry milk in PBS containing 0.2 % Tween-20 for 30 min at room temperature. After washing, the membranes were incubated overnight at 4°C with the rabbit polyclonal anti-MBNL1 antibody diluted 1:5,000 in PBS and 0.2% Tween 20. The membranes were then incubated for 1 h at room temperature with a

goat anti-rabbit HRP-conjugated secondary antibody, diluted 1:1,000 in PBS and 0.2 % Tween 20 (Abcam, Cambridge, UK). The immunocomplexes were finally revealed by the chemiluminescence reagent (ECL; Millipore, Billerica, MA, USA). Mouse monoclonal antibodies against actin (1:3,000, Sigma-Aldrich, St Louis, MO, USA) or glyceraldehyde-3-phosphate dehydrogenase (GADPH, routinely used in our laboratories as a standard for human samples) (Biogenesis, 1:10,000) were used as loading controls and revealed with a goat anti-mouse HRP-conjugated secondary antibody, diluted 1:3,000 in PBS and 0.2 % Tween 20 (Abcam). The mean ± standard error of the

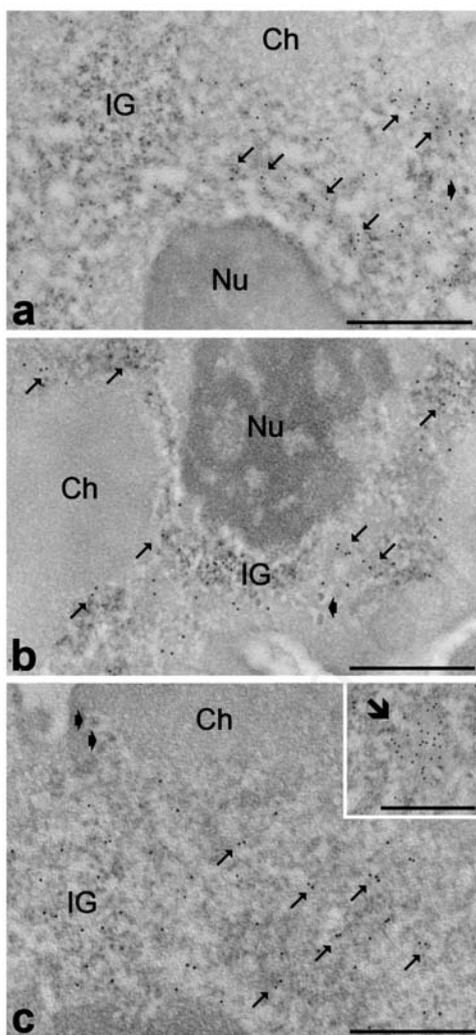mean (SE) was calculated, and statistical analysis was performed by the one-way ANOVA test. Statistical significance was set at P≤0.05.
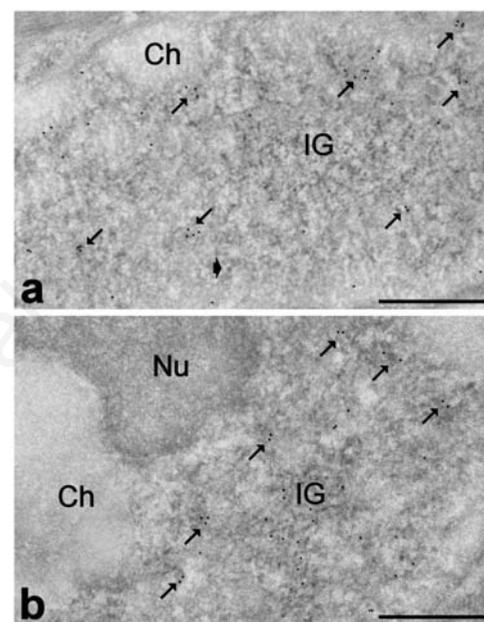
Results

Immunogold labelling

In the myonuclei of healthy human subjects (Figure 1a) and adult mice (Figure 2a) most of the anti-MBNL1 immunolabelling specifically occurred on PF, and only few gold grains were rarely observed on IG. In DM1 and DM2
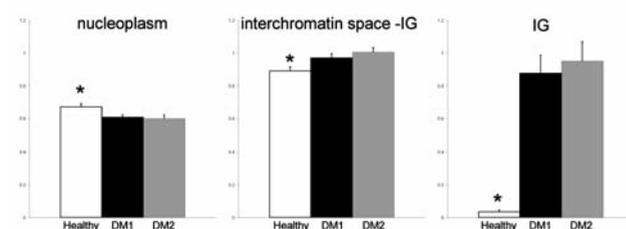
patients (Figure 1b,c) as well as in old mice (Figure 2b), the immunolabelling was present on PF and frequently also on IG. Moreover, in DM patients the signal was also found to accumulate in roundish domains (Figure 1c) corresponding to the *foci* described at fluorescence microscopy after RNA *in situ* hybridization or MBNL1 immuonolabelling.[18,21,35,52] The myonuclei we observed in DM patients or in old mice always showed the described reorganization of the RNP components, thus suggesting that such an alteration did affect all the myofibre nuclei. The gold labelling found on condensed chromatin and nucleoli was always negligible (Figures 1 and 2).

Figure 1. Myonuclei from healthy (a), DM1 (b) and DM2 (c) patients; anti-MBNL1 antibody. In healthy subjects (a), perichromatin fibrils (arrows) are specifically labelled, while the interchromatin granules (IG) are devoid of signal. In patients affected by DM1 (b) and DM2 (c), MBNL1 occurs both on perichromatin fibrils (arrows) and on interchromatin granule clusters (IG). In the inset (c) a roundish domain (thick arrow) corresponding to a *focus* shows a strong labelling for MBNL1. Condensed chromatin (Ch), nucleoli (Nu) and perichromatin granules (arrowheads) are not labelled. Scale bars: 500 nm.

Figure 2. Myonuclei from adult (a) and old (b) mice; anti-MBNL1 antibody. In adult animals (a), MBNL1 exclusively occurs on perichromatin fibrils (arrows), while the interchromatin granules (IG) are devoid of signal. Conversely, in old mice (b), both perichromatin fibrils (arrows) and interchromatin granule (IG) are labelled. Condensed chromatin (Ch), nucleoli (Nu) and perichromatin granules (arrowheads) are not labelled. Scale bars: 500 nm.

Figure 3. The histograms show the mean values±SE of the anti-MBNL1 labelling densities (gold grains/μm²) over different myonuclear compartments of healthy, DM1 and DM2 patients. Columns identified by asterisks are significantly different from each other.

Morphometric analyses demonstrated that the percentage of condensed chromatin was higher in the myonuclei of DM than of healthy subjects (means±SE values: healthy=24.40± 0.84%; DM1=35.98±0.78%; DM2=38.66± 0.76%); similar results were found for the mouse myonuclei with a larger fraction of condensed chromatin in old than in adult animals (means±SE values: adult=24.25±1.17%; old=36.06±1.80%).[41] The gold immunolabelling for MBNL1 was quantitatively assessed in the nucleolus, the nucleoplasm (excluding the DM *foci*), the *interchromatin space minus IG*, the IG clusters and the DM *foci*. In all samples the labelling on the nucleolus was extremely low (mean±SE: 0.008±0.004 gold particles/μm^2), similar to the background level (see below).

In human subjects (Figure 3) the labelling on the nucleoplasm was significantly lower in DM than in healthy patients; conversely, the immunogold values found on the *interchromatin space minus IG* or the IG clusters were significantly higher in DM patients in comparison to healthy subjects. In the mice, nucleoplasmic immunolabelling for MBNL1 (Figure 4) was not statistically different in adult and old animals (although the difference was close to the significance value, with P=0.06), whereas the values found on the *interchromatin space minus IG* and IG clusters were significantly higher in old than in adult individuals. In DM *foci* the mean ± SE values of immunolabelling were 209.41±43.46 gold particles/μm^2. Background values were negligible (0.006±0.003 gold particles/μm^2) in all immunolabelling experiments.

Western blotting analysis

The western blot analysis showed that in both DM patients and old mice skeletal muscle biopsies an increase of MBNL1 protein occurs, compared to healthy humans and adult mice,
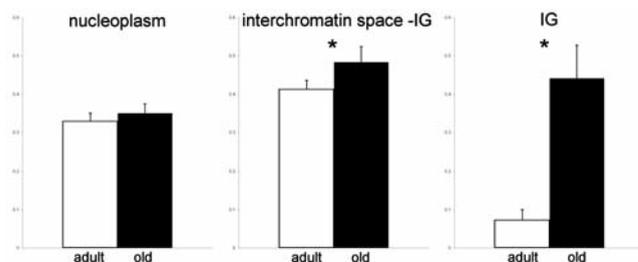
respectively (Figure 5a,b). This increase was found to be statistically significant by MBNL1 quantification normalized against the reference proteins (Figure 5 c,d). No statistically significant difference between DM1 and DM2 patients was found.

Discussion

In eukaryotic cells, nuclear RNPs are structural elements of the transcription and splicing machinery, and are always organized as morphologically recognizable structures called PF, IG and PG.[36] PF, in particular, are the sites of pre-mRNA transcription and co-transcriptional splicing as they contain nascent transcripts,[53,54] hnRNPs, snRNPs,[53] the non-snRNP SC35 splicing factor,[55] and 3' end processing.[56,57] IG are known to represent storage, assembly and recycling sites for transcription and splicing factors,[58,59] while their role in mRNA export remains somewhat controversial.[60,61] PG act as both vectors and storage sites of already spliced pre-mRNA.[36] The precise intranuclear localization of all these structures as well as their proper protein content are nec-

essary for the correct progress of mRNA maturation,[36,58] while their structural or compositional reorganization is a distinctive sign of impairment in this process.[38,62] Our findings demonstrate that, in skeletal myonuclei of both healthy adult humans and mice, MBNL1 usually occurs on PF, where it plays its post-transcriptional functions, and does not accumulate in any other nuclear domain. However, in muscles affected by DM or sarcopenia, MBNL1 is distributed not only on PF but frequently also on IG clusters. Therefore, in both DM and ageing, MBNL1 undergoes intranuclear relocation and moves to domains which are usually devoid of this protein in healthy adults. It is worth noting that an increase of transcription factors in IG clusters has been shown to occur during ageing in hepatocytes and neurons;[50,51,63] similarly, an accumulation of splicing factors (hnRNPs, snRNPs and SC35) in IG has been reported in different tissues under physiologically hypometabolic conditions.[64]

As expected, MBNL1 protein especially accumulates in the roundish domains corresponding to the peculiar *foci* of DM patients.[18,21,35,52] These RNP-containing aggregates are a typical marker of the DM genetic alterations, and have never been found in the myonuclei of healthy

Figure 5. Western blot analysis of the expression of MBNL1 and reference proteins in all healthy and DM patients (a) and in all adult and old mice (b). The histograms show the mean intensity level ± SE of MBNL1 protein, after normalization with the actin or GADPH intensity, in healthy and DM patients (c), and in adult and old mice (d). Asterisks indicate values significantly different from each other. No statistically significant difference was found between DM1 and DM2 patients.

Figure 4. The histograms show the mean values±SE of the anti-MBNL1 labelling densities (gold grains/μm^2) over different myonuclear compartments of adult and old mice. Asterisks indicate statistically significant difference.

adult or old individuals. However, recent results demonstrated that these *foci* increase in size and accumulate larger amounts of MBNL1 and RNA repeats in the myonuclei of DM2 patients during ageing as well as in senescing non-dividing cells in culture.[65] The quantitative study of immunolabelling adds interesting data on the reorganization of MBNL1 in the subnuclear domains in both dystrophic and sarcopenic muscles. In DM patients, the nucleoplasmic MBNL1 immunolabelling is significantly reduced in comparison to healthy subjects, consistent with the recruitment of the protein into the *foci* that leads to the depletion of free MBNL1 from the nucleoplasm.[24] However, MBNL1 accumulates in PF in the myonuclei affected by DM, as demonstrated by the larger amount of MBNL1 in the so-called *interchromatin space minus IG* domain (where PF are especially abundant), compared to healthy subjects. In addition, in DM muscles MBNL1 accumulates also in IG, as discussed above. This observation is not conflicting with the previously described nucleoplasmic depletion of MBNL1 in DM myonuclei observed after fluorescent immunolabelling:[24] by this approach, the fluorescent signals were measured over the whole nucleoplasmic surface, irrespective of their distribution in the different domains, whereas the ultrastructural gold immunolabelling allowed to localize the MBNL1 labelling in the interchromatin space subcompartments, and to detect the nucleoplasmic areas devoid of this protein (*i.e.*, condensed chromatin whose amount is significantly larger in the myonuclei of both DM1 and DM2 patients than of healthy subjects).

The amount of the whole nucleoplasmic MBNL1 immunolabelling was slightly (though not significantly) higher in old than in adult mice and, as much as it occurs in DM muscles, the *interchromatin space minus IG* and the IG clusters showed a significant accumulation of this protein in the nuclei of sarcopenic muscles from old individuals. Again, old myonuclei contain significantly higher amount of condensed chromatin than the adults.[37,39] Western blotting results support the immunohistochemical data, showing a significant increase in MBNL1 content both in dystrophic muscles (essentially depending on MBNL1 sequestration in the *foci*) and in sarcopenic muscles (where this mainly relates to the protein accumulation in RNP nuclear constituents).

Interestingly, it has recently been demonstrated[37] that in skeletal muscle biopsies from DM1 and DM2 patients, splicing and cleavage factors accumulate in their intranuclear functional sites (*i.e.*, PF) but also in the IG, while being massively sequestered in the RNP-containing *foci*. Similarly, factors involved in pre-mRNA post-transcriptional processing have been found to accumulate on PF in the nuclei

of sarcopenic muscles[39-41] as well as in other tissues (*e.g.*, liver, brain) during ageing.[50,51,63,66,67] As a consequence, the availability of this essential factor for alternative splicing would be reduced both in dystrophic and sarcopenic muscles, thus compromising the synthesis of several protein isoforms.[1,11,28-34] More generally, it could be hypothesised that the accumulation/delocalization of mRNA processing factors, including MBNL1, could hamper the functionality of the whole splicing machinery and slow down the intranuclear molecular trafficking, leading to a lower metabolic activity of myonuclei, consistent with the reduced protein synthesis observed in DM1 and DM2 myoblasts,[68,69] and the misregulated protein turnover resulting in a structural imbalance between protein synthesis and degradation in aged muscles.[70] It is worth noting that myoblasts from DM2 patients grown in culture show cell-senescence structural alterations and impairment of the pre-mRNA maturation pathways much earlier than the myoblasts from healthy subjects.[71] The skeletal muscle of DM patients seems therefore to share intriguing similarities with the muscle from aged mammals, with special reference to the alterations in the nuclear RNP-containing structures involved in pre-mRNA transcription and splicing. These nuclear similarities may account for the similar muscle tissue phenotype (myofibre atrophy, fibre size variability and centrally located nuclei) and, in general, for the aging-reminiscent phenotype especially observed in patients affected by the more severe DM1 form which shows numerous degenerative adult-onset disorders (*e.g.*, muscle weakness and atrophy, bilateral ocular cataracts, type 2 diabetes mellitus, cardiomyopathy, testicular atrophy, immune deficiency).[72]

As a more general remark, the present results on the subnuclear redistribution of MBNL1 in DM and in sarcopenic muscles confirm and extend previous observations indicating the reorganization of the nuclear RNP components as a univocal cytological marker of muscle cell dysfunctions. Comparative studies are therefore encouraged in the attempt to detect common molecular mechanisms at the basis of skeletal muscle wasting under physiological or pathological conditions.

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