Muscle cell differentiation and development pathway defects in Emery-Dreifuss muscular dystrophy

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Abstract

Emery-Dreifuss muscular dystrophy (EDMD) is a rare genetic disorder characterised by the early development of muscle contractures, progressive muscle weakness, and heart abnormalities. The latter may result in serious complications, or in severe cases, sudden death. Currently, there are very few effective treatment options available for EDMD and so there is a high clinical need for new therapies. Various genetic mutations have been identified in the development and causation of EDMD, each encoding proteins that are components of the Linker of Nucleoskeleton and Cytoskeleton (LINC) complex, which spans the nuclear envelope and serves to connect the nuclear lamina to the cytoskeleton. Within this review, we examine how mutations in the genes encoding these proteins, including lamins A/C, emerin, nesprins 1/2, FHL1, and SUN1/2 lead to muscle cell differentiation and development pathway defects. Further work to identify conserved molecular pathways downstream of these defective proteins may reveal potential targets for therapy design.

1. Introduction

Emery-Dreifuss muscular dystrophy (EDMD) is a rare genetic neuromuscular disorder with an estimated world-wide incidence of 1 in 100,000 [1]. The disease is characterised in the early stages by muscle contractures that usually become noticeable during childhood or adolescence[2], accompanied by muscle weakness and wasting that worsens over time. Almost all people with EDMD develop heart abnormalities by adolescence [3]. These typically present as atrioventricular conduction block [2,4,5], but in some cases, dilated cardiomyopathy may gradually develop, which can be further complicated by ventricular tachydysrhythmias [3,5]. Very few treatment options exist for EDMD. Surgery can be used to release contractures, and the implantation of a cardiac pacemaker when symptomatic bradycardia or conduction defects are identified can decrease the risk of potentially life-threatening arrhythmias [6]. In more severe cases of EDMD, pacemakers are often not sufficient to eliminate the cardiac risks of EDMD, and so implantation of an ICD (implantable cardioverter-defibrillator) is recommended [6]. Heart transplants are also often needed in patients with AD-EDMD with cardiomyopathy [7]. Consequently, there is a great need for new, innovative therapies for the treatment of EDMD.

There are at least three modes of inheritance of EDMD. Mutations in *EMD*, encoding the inner nuclear membrane (INM) protein, emerin, give rise to X-linked EDMD [8]. *LMNA* mutations, leading to disruptions in nuclear lamins A and C, are responsible for both the autosomal dominant and, more rarely, the autosomal recessive forms of the condition [9,10]. In addition to *EMD* and *LMNA*, other mutant genes and proteins have been linked to EDMD. Nuclear envelope (NE) proteins nesprins-1/2, encoded by *SYNE1* and *SYNE2*, and SUN1 and SUN2 [11] have been associated with the development of EDMD [12], and

mutations in *FHL1* [13], a gene that encodes multiple FHL1 protein isoforms, particularly isoform FHL1B (also recently identified as a NE protein), is misregulated in EDMD [14]. More recently, mutations in *TMEM43*, the gene encoding LUMA, have also been linked to EDMD [15,16]. Each of the proteins encoded by these genes are components of the linker of nucleoskeleton and cytoskeleton (LINC) complex that spans the nuclear envelope and functions to connect the nuclear lamina (NL) to the cytoskeleton [17]. This is with exception to FHL1, which has not yet been identified as part of the LINC complex, but it has been shown to interact with lamin A/C and emerin [14]. The central components of the LINC complex are nesprin-1 giant and Sad1 and UNC-84 (SUN) domain proteins situated at the ONM and INM, respectively [18]. The SUN and Klarsicht/Anc-1/Syne Homology (KASH) domains of the respective proteins interact in the perinuclear space to form a bridge that spans the INM, perinuclear space and ONM [18]. It is not yet known if each SUN trimer attaches to three molecules of a single nesprin isoform, or to mixtures of nesprin isoforms, possibly including both nesprin-1 and nesprin-2 molecules [19]. The N-termini of SUN proteins, SUN1 and SUN2, interact with lamin A/C at the NL and anchor the LINC complex at the NE, whilst nesprin-1 giant binds the actin cytoskeleton through its N-terminal calponin homology domain (Figure 1) [20,21].

[Figure 1]

Identification of mutations in nuclear envelope genes that are involved in EDMD has been influential in guiding the direction of research and has allowed the development of important research tools. Correcting the genetic defect is one approach to therapy for EDMD but by the time severe symptoms have developed, and a diagnosis is made, it may be

too late for this to be fully effective. The issue is further complicated by the fact that although EDMD disease onset is typically during childhood or early adolescence, this, along with the severity and phenotype of the disease, is often variable between individuals with little obvious relationship to the causative mutation [22]. An alternative approach is to identify - and tailor - therapies towards conserved features of the disease that could, in combination with gene-targeted therapy, offer maximum therapeutic benefit to all EDMD patients. Indeed, this strategy is gaining significant momentum for another inherited neuromuscular disease called spinal muscular atrophy (SMA). In ~95% of cases, SMA is caused by homozygous loss of *SMN1* gene, resulting in reduced levels of the survival of motor neuron (SMN) protein [23]. Efforts to increase SMN protein levels using Nusinersen (Spinraza^{TM}), an antisense oligonucleotide drug, or ZolgensmaTM (previously known as AVXS-101), an adeno-associated viral-based gene replacement therapy, have been fruitful but none of these strategies show complete efficiency in patients [24–27]. Work is now underway to find alternative therapeutic strategies to offer in combination with SMN-targeted therapy to maximise therapeutic benefit for SMA patients [28]. The ability to identify alternative therapeutic targets, however, requires an understanding of the molecular pathways acting downstream from the defective gene(s) to modulate disease pathogenesis. Some progress has been made to understand these pathways in EDMD, and in this review, we examine an emerging body of research that supports a role for defects in muscle cell differentiation and development pathways as conserved features in EDMD. Particular attention is given to lamins A/C, emerin and nesprins 1 and 2, and how mutations in each of these might contribute to differentiation and development pathway defects (as summarised in Table 1).

[Suggested position for Table 1]

2. Emerin

Loss-of-function mutations or null-mutations of the *EMD* gene encoding emerin, an integral membrane protein of the inner nuclear membrane (INM), cause the X-linked form of Emery-Dreifuss muscular dystrophy (EDMD) [8]. Emerin is proposed to play an important role in the regulation of myogenic differentiation by two potential mechanisms: the regulation of intracellular signalling cascades and, like lamin A/C, the regulation of chromatin architecture [29].

2.1. Emerin is involved in regulation of myogenic differentiation signalling pathways

Growing evidence suggests emerin is involved in regulating the expression or activity of key components of signalling pathways that are important for myogenic differentiation. Expression profiling of mRNA and miRNA in *Emd*-null mouse myogenic progenitors revealed disruptions to the Wnt, IGF-1, TGF-ß, and Notch signalling pathways, which are all important in regulating myogenic differentiation and muscle regeneration [30–33]. These pathways have well-established roles in maintaining satellite cell quiescence, satellite cell activation and myogenic differentiation after injury [34]. The JNK, p38 MAPK, ERK and NF-κB signalling pathways were also found to be disrupted in cardiomyocytes derived from $Emd^{-/y}$ mice [35] and the ERK pathway was upregulated in emerin-knockdown HeLa and C2C12 cells [36]. C2C12 myoblasts with reduced levels of emerin also had impaired differentiation, which was partially rescued by treatment with ERK inhibitor U0126 [37].

ERK inhibition is required early during differentiation to initiate myogenesis, and early and late ERK activity is crucial for proper myogenic differentiation [26]. It is also known that temporary ERK inhibition coordinated with p38 MAPK activation is essential for controlling the coordinated temporal expression of differentiation genes during myogenic differentiation [38]. p38 MAPK is an *Myod1* activator, and sustained levels of p38 MAPK are required for the formation of MyHC-positive myotubes [39]. Moreover, myogenic differentiation is accelerated in myoblasts expressing constitutively active p38 MAPK [26]. *Emd*-null H2K myogenic progenitors have increased levels of phosphorylated p38 MAPK, however inhibition of p38 MAPK arrests differentiation [26]. This suggests that maintaining the correct levels of phosphorylated p38 MAPK within a narrow range appears to be required for proper differentiation.

Lim-domain only 7 (Lmo7) directly binds emerin *in vitro*, associates with emerin *in vivo,* and is a transcription activator *in vivo*, as initially demonstrated in a HeLa cell line [40]. Lmo7 regulates emerin gene expression but requires emerin to localise efficiently in the nucleus, and it is also functionally inhibited by binding to emerin [40]. Lmo7 is necessary for proper myoblast differentiation, as evidenced by findings that C2C12 myoblasts with downregulated Lmo7 exhibit reduced expression of Pax3, Pax7, Myf5 and MyoD, whilst overexpression of GFP-Lmo7 increased the expression of MyoD, Pax3, and Myf5 [41]. In C2C12 myoblasts and *in vitro*, it was shown that Lmo7 bound the *Pax3, MyoD* and *Myd5* promoters [41]. Additionally, it was discovered that emerin binding to Lmo7 inhibited Lmo7 binding to - and activating - the *MyoD* and *Pax3* promotors [41]. These results suggest that the functional interaction between emerin and Lmo7 is crucial for temporally regulating the expression of key myogenic differentiation genes [41]. Furthermore, gene-trap mediated

deletion of a single Lmo7 isoform in mice causes myopathic phenotypes similar to those seen in other EDMD mouse models [42]. These "Lmo7-null" mice were found to have growth retardation, decreased fibre size, and impaired skeletal muscle and cardiac function, together with lower levels of phosphorylated retinoblastoma (Rb), extracellular signal-regulated kinase, and c-Jun N-terminal kinase (JNK)[42]. In another study, novel Lmo7-null (*Lmo7*$^{-/-}$) mice were generated with deletion of all three isoforms of Lmo7. Conversely, these *Lmo7*$^{-/-}$ mice were found to have no abnormalities in skeletal muscle morphology, function, or regeneration, and cardiac function was also normal. Ablation of *Lmo7* in the dystrophin-deficient *mdx* mouse model also had no effect on the myopathy and muscle regeneration exhibited in *mdx* mice. These results indicate that Lmo7 is dispensable for skeletal muscle function, but given the contradictions in the literature, future work is clearly warranted.

A bioinformatic array analysis of regenerating *Emd* null muscle derived from *Emd*$^{-/y}$ mice revealed abnormalities in cell-cycle parameters and delayed myogenic differentiation associated with perturbations to transcriptional pathways regulated by retinoblastoma (*Rb1*) and *MyoD* [43]. Temporal activation of MyoD transcriptional targets was significantly delayed, whereas targets of the Rb1/E2F transcriptional repressor complex remained inappropriately active [43]. The inappropriate modulation of Rb1/MyoD transcriptional targets was associated with the up-regulation of Rb1, MyoD and their co-activators/repressors transcripts [43]. This suggests an over-compensatory effort to overcome a molecular block to differentiation. Analysis of Rb1 phosphorylation states showed prolonged hyper-phosphorylation at key development stages in *Emd* null myogenic cells, both *in vivo* and *in vitro* [43]. Alterations in Rb1 transcriptional regulatory pathways have also been observed in patients with X-linked EDMD[43].

Emd-null mice appear to display a mild phenotype compared to humans with EDMD. They have been shown to have normal growth and show no obvious muscle degeneration, joint contractures or cardiac symptoms [43,44]. Despite this, studies using emerin-null mice have, however, revealed molecular defects including dysfunctional Wnt/β catenin signalling [45] and, as mentioned previously, irregularities in the MyoD/Rb pathways [43]. It is not unusual for animal models to show milder symptoms to the corresponding human disease, and in this case, this may be a due to interspecies differences in possible shared functions of INM proteins, and other INM proteins compensating for the loss of emerin [44]. In contrast to the *Emd*-null mice, *Lmna*-deficient mice have dystrophic muscle [43]. In *Lmna*-null muscle, different perturbations in expression of MyoD targets have been found, which suggests different compensatory mechanisms may exist between *Lmna*-null and *Emd*-null muscle [43].

2.2. Emerin is required for correct genomic reorganisation during myogenesis

Increasing evidence shows that emerin has a role in establishing, maintaining, or recruiting repressed chromatin to the nuclear lamina at the nuclear envelope [29]. Emerin binds directly to chromatin regulatory complexes containing BAF, or histone deacetylases [29,46]. BAF recruits emerin to chromatin during nuclear assembly and also mediates chromatin decondensation [46,47]. Emerin binds to histone deacetylase 3 (HDAC3), a component of the nuclear receptor co-repressor (NCoR) complex [48], which localises to gene regulatory regions and represses the transcription of genes under their control [48]. Binding of emerin activates HDAC3 activity, recruiting it to the nuclear envelope, as demonstrated *in vitro* using C2C12 lysates and C2C12 myoblasts [48]. This interaction coordinates the

spatiotemporal nuclear envelope localisation of genomic regions containing *Myf5* and *Myod1*, myogenic regulatory factors involved in regulating muscle differentiation, and *Pax7*, a transcription factor that regulates muscle precursor cell proliferation during myogenesis to ensure differentiation proceeds normally (as observed in murine myogenic progenitors) [49]. Loss of emerin disrupts this genomic reorganisation, which, interestingly, is rescued by treatment with theophylline, a methylxanthine drug used in therapy for respiratory diseases that stimulates HDAC3 activity [29]. LAP2β also interacts with HDAC3 and induces H4 deacetylation, contributing to LAD formation [50]. It has been suggested that theophylline's action in rescuing genomic organisation and myogenic differentiation in emerin-null cells is the result of an increase in the association of HDAC3 with LAP2β, which rescues the coordinated temporal sequestration and silencing of promoters to temporally regulate the differentiation transcriptional program [29].

Theophylline has also been shown to rescue myotube formation in emerin-null myogenic progenitor cells, suggesting HDAC3 activity is important for later stages of myogenic differentiation [29]. Thus, emerin regulation of HDAC3 activity may control the coordinated temporal expression of genes important for cell fusion or myotube maturation. Alternatively, emerin regulation of HDAC3 activity may be necessary to coordinate the expression of both early and late differentiation genes at an earlier stage of differentiation, with the defect not being apparent until later in differentiation [29]. Consistent with these observations, HDAC3-specific inhibitor RGFP966 blocks myosin heavy chain (MyHC) expression and fusion in both differentiating wild-type and emerin-null H2K myogenic progenitors [29]. MyHC is a constituent of the myosin protein complex (comprised of two heavy chains and two light chains twisted around each other) that forms the molecular

motor that drives muscle contraction [51]. MyHCs are specifically responsible for the power stroke movement that breaks down ATP, converting it to mechanical energy that pulls the actin filament across the myosin filament [51]. It has been predicted that HDAC3 activity is required for the transition from proliferating myogenic progenitors to differentiating myoblasts by repressing the expression of genes that are important for myogenic progenitor proliferation and induction of the differentiation gene program, as inhibition of HDAC3 blocks this transition [29]. Additionally, HDAC3 activity may be required for myotube formation.

2.3 Loss of emerin may affect the integrity of the LINC complex

It is currently unknown whether emerin mutations have any effect on the LINC complex function; however, as emerin is known to interact with other components of the LINC complex, it is certainly a possibility. Emerin has been shown to directly bind to lamin A [52,53] and using transfection of lamin A/C-deficient fibroblasts, it was found that mutations affecting the tail domain of lamin A work by directly impairing emerin interaction (whereas mutations in the rod region cause defective lamina assembly) [54]. In human skin fibroblasts, it has also been demonstrated that emerin anchors nesprin-2 giant at the nuclear envelope [55].

3. Lamin A/C

The nuclear lamina (NL) is a filamentous protein network that underlies the inner nuclear membrane (INM) [56]. Major components of the NL are type V intermediate filaments, A- and B-type lamins[56]. A-type lamins, lamins A and C, are encoded by a single gene known as *LMNA,* and mutations in *LMNA* are associated with many different diseases, including

autosomal-dominant Emery-Dreifuss muscular dystrophy (AD-EDMD) [9]. These diseases, collectively termed laminopathies, are associated with a wide range of phenotypes, including neuropathies, muscular dystrophies and lipodystrophies [57]. Lamins A/C is expressed in almost all differentiated cells and tissues, though most laminopathies appear to affect only one or a few tissue types [58]. Two hypotheses attempt to explain this. According to the "structural" hypothesis, mutations in A-type lamins alter the integrity of the NL [59][60]. This leads to structural weakness, finally resulting in the nucleus being unable to resist high mechanical stress within cells [59][60]. This mainly affects tissues exposed to strong mechanical tension, which is particularly pertinent within muscle. The alternative "gene-expression" hypothesis is based on interactions between the NL and chromatin and between the NL and transcription factors and chromatin modifiers [61]. This theory assumes that mutations in *LMNA* alter the regulation of gene expression during differentiation. There is accumulating evidence that these hypotheses may not be mutually exclusive; the consequences of which include perturbation of muscle cell development and differentiation pathways, as discussed in the sections that follow.

3.1. LMNA is important for correct positioning of myonuclei

Skeletal muscle is composed of multinucleated fibres formed by the fusion of mononucleated cells. Approximately four to eight nuclei, amongst the hundreds of nuclei in a muscle fibre, are specialised for the transcription of the components of the synapse at the neuromuscular junction (NMJ) [62][63]. These "synaptic nuclei" are recruited into the postsynaptic membrane area during muscle differentiation, and their proper positioning is important for NMJ establishment and maintenance [64]. The positioning of synaptic nuclei

at the NMJ involves an interaction between the cytoskeleton and various nuclear envelope (NE) proteins, including nesprin and SUN proteins [65][66]. The C-terminal KASH domain of nesprin proteins interacts with SUN proteins, which span the INM and physically interact with A-type lamins, establishing a continuous physical link between the NL and the cytoskeleton [67][68][17][69]. Previous research has strongly suggested that NMJ defects contribute to the AD-EDMD disease phenotype. Muscle from two established mouse models of *Lmna* mutations, $Lmna^{H222P/H222P}$ and $Lmna^{-/-}$ (referred to as models of AD-EDMD [70]), exhibited altered innervation patterns and abnormal NMJ morphology [70]. Consequently, these mice show signs of innervation defects including differential expression of electrical activity-dependent genes and altered epigenetic chromatin modifications [70]. These same molecular have been observed in muscle from AD-EDMD patients with *LMNA* mutations[70]. At the molecular level, several NE proteins involved in proper positioning of nuclei near the synapse were mislocalised in the AD-EDMD mouse models, and recruitment of synaptic nuclei was impaired [70].

Altered anchorage of myonuclei and myonuclear clustering have been linked to the pathogenesis of muscle disorders, including those associated with mutations in NE proteins [71][11][72]. Disrupted nuclear positioning has been observed in a multitude of muscle disorders and results in ineffective muscle contraction. Nuclei are normally situated at the periphery of myofibers, just below the plasma membrane. This positioning is a hallmark of skeletal muscle, but the function of this structural trait is still uncertain [73]. Many NE and LINC complex proteins already have well-established roles in nuclear positioning in muscle, and it has been speculated that a protein complex that is involved in centrosome interplay during nuclear movement exists at the nuclear poles of differentiating myoblasts which may

be disrupted in muscle diseases of the NE [74]. Lamin A/C binds to several NE components, including the INM protein, spindle associated membrane protein 1 (Samp1), which is believed to be involved in nuclear movement and is necessary for muscle cell differentiation [74]. It was demonstrated that prelamin A farnesylation impairment causes loss of Samp1 from the nuclear poles in myotubes derived from healthy subjects, suggesting that farnesylated prelamin A is required to anchor Samp1 in the nuclear poles of differentiated muscle cells [74]. In the nuclei of myoblasts and myotubes derived from an AD-EDMD patient harbouring an H506P *LMNA* mutation, Samp1 was also absent from most of the nuclear poles [74]. These results suggest that defects in Samp1 and its binding partners could impair nuclear movement occurring during myoblast differentiation in AD-EDMD. In another study, however, Samp1 was found to be correctly localised [75], which may mean Samp1 mislocalisation is dependent on the specific *LMNA* mutation. The presence of other proteins at the nuclear poles, such as nesprin-1 or nesprin-1α2 that are also relevant for correct nuclear positioning, were not studied but would be useful to investigate in future work.

3.2. The role of LMNA in myogenic differentiation

In *Lmna*$^{-/-}$ H-2K myoblasts, two major alterations in the bone morphogenetic protein (BMP) pathway have been identified: Bmp4 downregulation and Smad6 overexpression [76]. These same pathway defects were found to be present in myoblasts from patients carrying different *LMNA* mutations[76]. Bmp4 downregulation in combination with Smad6 overexpression lead to premature differentiation in *Lmna*$^{-/-}$ H-2K myoblasts, which was rescued by the downregulation of Smad6 [76]. Receptor-regulated Smads (R-Smads), Smad1, Smad5 and Smad8 (pSmad1/5/8), are phosphorylated in response to the binding

of Bmp4 to type 1 and type 2 transmembrane receptors and can then form a complex with Smad4 (Co- Smad), allowing their translocation into the nucleus to induce the transcription of target genes such as *Id1* and *Id2* [77]. Id protein expression prevents the premature activation of differentiation factors, hence preventing premature muscle differentiation [77]. Alternatively, phosphorylated R-Smads may interact with Smad6, which has an inhibitory role on the BMP pathway and avoids the expression of target genes [77]. The way by which lamins A/C may regulate Bmp4 and Smad6 expression is currently unclear. However, Smads are also components of the TGFβ pathway, and alterations in this pathway have been previously described in the same model of *Lmna*$^{-/-}$ myoblasts, where increased nuclear levels of Smad2/3 have been observed, causing delayed activation of satellite cells [76]. Similarly to the BMP4 pathway, when Smad2 and Smad3 are phosphorylated in response to the binding of a ligand to its receptor, they bind Smad4 and translocate into the nucleus to activate target genes expression [77,78]. In a similar way to Smad6, Smad7 competes with Smad4 to prevent Smad2/3 intranuclear translocation [79,80]. It has been suggested that the competition between Smad6 and Smad4 to interact with pSmad1/5/8 could lead to increased availability of Smad4 to interact with pSmad2/3 [76]. Consequently, changes in Smad6 expression would indirectly affect the TGFβ pathway by increasing the intranuclear translocation of Smad4-pSmad2/3. The effects of overexpression of Smad6 in *Lmna*$^{-/-}$ myoblasts might, therefore, be due to an imbalance between BMP4 and TGFβ pathways [76].

Three key cellular processes are involved in myogenic differentiation: the inactivation of pluripotency programs (the Oct4-Nanog-Sox2 program), exit from the cell cycle (involving cyclin-dependent kinase 1, and retinoblastoma 1), and induction of myogenesis (by master

transcriptional regulators, MyoD and myogenin) [81]. *LMNA* plays a well-recognised role in the cell cycle and is transcriptionally induced at both the point of exit from the cell cycle, and during the onset of terminal differentiation of myoblasts into myotubes [82][83]. There is also preliminary evidence, from an *in-vitro* study of HeLa cells transfected with *MyoD*, that *LMNA* binds (either directly or indirectly) to MyoD, but this requires further investigation [82]. Expression of R453W *LMNA* in C2C12 cells impaired activation of the myogenin gene (*Myog)* and maintained a repressive chromatin state on the *Myog* promoter upon induction of differentiation [84]. Furthermore, expression of R453W *LMNA* in undifferentiated C2C12 cells redistributed H3K9me3 from pericentric heterochromatin [84] (i.e. heterochromatin that is located adjacent to the CENP-A rich centromere "central core" and is characterised by H3K9me3, a modified histone [85]). H3K9me3 contributes to gene regulation by forming repressive domains on chromosomes, preventing transcription factor binding [85]. Conversely, the aggregation of pericentric heterochromatin into chromocentres is a hallmark of myogenic differentiation [84][86].

3.3. The involvement of LMNA in regulation of chromatin structure and function

In myoblasts taken from a patient with severe AD-EDMD caused by a p.R545C *LMNA* mutation, the distribution of histone H3 trimethylated at lysine 27 (H327me3) and phosphorylated RNA polymerase II (markers of inactive and active chromatin domains, respectively) were altered [87]. These findings support the theory that *LMNA* mutations may affect transcription regulation in cells by imposing changes in the structure of chromatin, and by modulating the distribution or function of factors regulating gene expression. Patient myoblasts with the p.R545C *LMNA* mutation also presented an abnormally high level of senescence in *ex vivo* culture [87]. Cellular senescence has

previously been correlated to proteasome inhibition [88]. In healthy individuals, proteins that are no longer required or that are damaged are degraded by 20S proteasomes, preventing the accumulation of misfolded proteins and protein aggregation [88]. In contrast, in aging patients, the accumulation of large quantities of oxidised proteins hinders proteasome function [89]. In myoblasts with the p.R545C *LMNA* mutation, data showed defective proteasome function, indicating that the p.R545C *LMNA* mutation may also contribute to premature senescence in AD-EDMD patient myoblasts [87].

The nuclear lamina interacts with chromatin at genomic regions called lamina-associated domains (LADs), which are heterochromatic (transcriptionally repressed) domains [81]. The NL interacts with the INM and DNA, providing structural support and regulating DNA replication, transcription, and cell division [81]. There is accumulating evidence that lamin A is involved in the epigenomic regulation of chromatin via an interaction with LADs. By mapping the euchromatin (transcriptionally active) transitions during muscle cell differentiation, *LMNA* mutations have been observed to disrupt the normal euchromatic-heterochromatic (epigenomic) transitions at the NE during the differentiation of murine H2K cells and fibroblasts from an EDMD patient with a p.H222P *LMNA* mutation [81]. During muscle formation, normal heterochromatin to euchromatin transitions that take place at myogenic loci drive muscle differentiation [81]. Observed perturbations of the epigenomic transitions included exit from pluripotency and cell cycle programs, as well as the induction of myogenic loci [81]. Furthermore, muscle biopsies from EDMD patients with gain- or change-of-function *LMNA* gene mutations showed an inappropriate loss of heterochromatin formation at the *Sox2* pluripotency locus, which continued to be expressed and inhibited myogenic differentiation in human myoblasts [81]. The results of this study suggest that

EDMD could be caused, at least in part, by aberrant LADS that disrupt developmental epigenetic programming.

4. Nesprin-1/2

Mutations in the *SYNE1* and *SYNE2* genes, encoding nesprin-1 and -2, have been identified in association with EDMD [12]. Extensive alternative transcription and splicing of *SYNE1* and *SYNE2* generates multiple nesprin-1/2 isoforms that vary greatly in size and exhibit multiple subcellular localisations [90]. In fact, prior to the discovery of giant nesprin-1/2, the smaller nesprin-1 isoform, nesprin-1α2 was first identified. Giant nesprin-1/2 generally consists of a C-terminal KASH domain that is targeted to the nuclear envelope (NE), a N-terminal paired calponin homology (CH) domain that binds the actin cytoskeleton, and a central rod domain containing multiple spectrin repeats (SRs) that link the CH and KASH domains and mediate protein-protein interactions (see figure 2) [91]. A highly conserved 18 amino acid sequence "Star" domain is also located between spectrin repeats 71 and 72 of giant nesprin-1/2 [92], [93], and downstream of the Star domain is another highly conserved 23 amino acid sequence encoded by an alternatively spliced exon "DV23"[92,94]. Giant nesprin-1/2 isoforms localise at the outer nuclear membrane (ONM) and interact with SUN1/2 at the perinuclear space, forming the LINC complex and connecting the nucleus to the actin skeleton [95].

[Figure 2]

4.1. Like lamin A, nesprin 1 and 2 are important for nuclear positioning in myotubes

During skeletal muscle differentiation, a dynamic change of both giant nesprin-1/2 and smaller isoforms occurs. As immature human muscle fibres begin to mature *in vivo,* nesprin-2 was found to partly replace nesprin-1 at the NE, whilst shorter nesprin isoforms became dominant [55]. Nesprin-1α2, the short nesprin-1 isoform, is only found in skeletal and cardiac muscle [94]. The N-terminal sequence of nesprin-1α2 is different to nesprin-1 giant, but the two structures have identical to the C-terminal regions, both containing the star region [92]. Nesprin-1α2 mRNA and protein expression is turned during myogenesis and dynamically controlled, suggesting the isoform may be involved during early myofiber formation [96]. Unlike nesprin-1 giant, which is present throughout muscle development, nesprin-1α2 appears after myoblast differentiation into early myotubes, remaining at high levels in immature muscle fibres, but then seems to decrease in expression in most mature adult muscle fibres [96]. Additionally, the expression levels of myogenic regulatory factors, including MyoD, myogenin and MyHC, microtubule motor protein KLC-1/2 as well as centrosomal proteins including Akap450, pericentriolar material 1 and pericentrin, are all increased during myoblast differentiation in association with upregulated nesprin-1α2 in differentiated myotubes [94,97,98]. As mentioned previously, correct nuclear movement and positioning is necessary for proper muscle cell development. Nuclear movement during myofiber maturation is a step-by-step process involving five types of nuclear events: centration, alignment, spreading, peripheral movement and anchoring [75]. Apart from the neuromuscular junction (NMJ) and myotendinous junction (MTJ) which contain clustered nuclei, the nuclei of myofibers are uniformly distributed [17,99]. Nuclear movement is primarily driven by cytoskeletal networks of microtubules, involving a connection between the cytoskeleton and the NE, with certain nuclear migrations requiring the LINC complex [73,95,98]. The microtubule motor protein kinesin-1 is a heterotetramer, composed of a

dimer of kinesin heavy chains (KHCs) and two regulatory kinesin light chains (KLCs)[100]. Nesprin-1 and -2 have both been shown to bind to and anchor KLC-1/2 to the nucleus through their LEWD motif, located at the star domain; an interaction which is essential for nuclear distribution during skeletal muscle cell differentiation [100]. It has been demonstrated that some nesprin-1 mutations disrupt this interaction [98]. Furthermore, depletion of KLC-1 results in nuclear clustering, whereas depletion of KLC-2, a dominant muscle isoform, caused reductions in MyHC levels and the fusion index in myotubes [98]. In addition, a recent study has suggested a function for nesprin-1α2 in the specific localisation of skeletal muscle nuclei at NMJ mediated by kinesins [19]. This study indicates that the primary role of nesprin-1α2 is at the outer nuclear membrane [19], and argues against previous evidence for an additional function at the inner nuclear membrane [101]. Nesprin-1α2 has also been shown to be involved in facilitating the movement of the microtubule-organising centre (MOC) from the centrosome in myoblasts, to the cytoplasmic surface of the NE in myotubes through recruiting Akap450 to the NE [97,102]. The recruitment of centrosomal proteins to the NE has been proposed to be responsible for microtubule nucleation from the nucleus in differentiated myoblasts and myotubes and subsequent nuclear positioning [97,103]. These data suggest that nesprin-1/-2 isoforms are important for nuclear positioning in myotubes [95].

Various mouse models have been generated to further understand the role of nesprins-1/2 in nesprin-related muscle diseases. Skeletal muscle defects have been observed in nesprin mouse models including nesprin-1 or 2 KASH overexpression, nesprin-1 or 2 KO and nesprin-1α_2 KO mice [65,66,104–106]. A reoccurring observation in these mice was that they displayed abnormal nuclear positioning in myofibers, and showed increased variability of

muscle fibre size [95][106]. Myonuclei clusters were found to be present in the longitudinal section of muscle fibres [65,66,106] and muscle fibre cross-sections contained centralised myonuclei [104,106]. Myonuclei detached upon removal of the sarcolemma from muscle fibres, and in nesprin-1 KO mice, nuclear aspect ratio (length divided by width) changed less when muscle fibres were subjected to mechanical strain when compared to wild type, indicating defective nuclear anchorage and decreased force transmission [95,106]. Furthermore, muscle fibre function appears to be compromised in nesprin-1 mutant mice as they display growth retardation, increased variability in body weight and decreased survival rate [105,106].

4.2. Nesprin-1 may regulate expression of muscle-specific genes

C2C12 myoblast cells were generated harbouring novel nesprin-1 mutants identified in patients with dilated cardiomyopathy (i.e. R8272Q, S8381C and N8406K, as well as dominant negative-1KASH) [98]. C2C12 cells expressing mutant R8272Q or dominant negative-1KASH were found to have a lower capacity to differentiate and form multinucleated cells [98]. Levels of myogenin and MyHC genes were also dramatically reduced [98]. It was observed that nesprin-1α2 was expressed in the initial stage where C2C12 myoblasts were converted into myotubes, and promoted myoblast differentiation during myotube formation [98]. The mutants reduced or abolished these effects, suggesting that nesprin-1 mutations do not alter myoblast proliferation, but may impair their capacity to express muscle-specific genes (i.e. myogenin and MyHC), resulting in impaired myoblast fusion. These data suggest that the regulation of myogenin and expression of MyHC are influenced by the LINC complex [98]. More specifically, nesprin mutants may fail to build a functional scaffold and/or maintain chromatin compartmentalisation with lamin A/C,

leading to an alteration in heterochromatin [98]. This could cause defects in terminal differentiation initiation, resulting in decreased or delayed expression of myogenin and MyHC and causing delayed differentiation.

5. FHL1

Mutations in the gene encoding the four-and-a-half LIM domain protein 1 (FHL1) have also been associated with the pathogenesis of EDMD [13], although it remains to be clarified whether mutations in FHL1 cause skeletal muscle remodelling as a result of gain or loss of FHL1 function [107]. There are spliced variants of FHL1: FHL1A, FHL1B and FHL1C, which are composed of a half LIM followed by four, three, and two full LIM domains, respectively. FHL1 protein is not a nuclear envelope protein, but FHL1A is predominantly expressed in striated muscles [108,109], and the other two less abundant isoforms, FHL1B and FHL1C, are also expressed in striated muscles and in the brain and testis [110,111].

To evaluate FHL1 loss-of-function effects on skeletal muscle, mice lacking global FHL1 expression were developed (FHL1-null mice). Histological and functional analyses of muscle derived from FHL-1 null mice demonstrated that they develop an age-dependent myopathy associated with myofibrillar and intermyofibrillar (mitochondrial and sarcoplasmic reticulum) disorganisation, impaired muscle oxidative capacity and increased autophagic activity [107]. Furthermore, analysis of primary myoblasts isolated from FHL-1 null muscle demonstrated early muscle fibre differentiation and maturation defects, which could be rescued by re-expression of the FHL1A isoform [107]. These results highlight that the FHL1A isoform is necessary for proper muscle fibre differentiation and maturation *in vitro*. The majority of FHL1 mutations identified in EDMD patients, however, are missense mutations

or in-frame deletions [13]. In these cases, mutant phenotypes may result from loss-of-function alone (FHL1 downregulation along with impaired protein partner binding), or possibly from simultaneous loss of FHL1 function and gain of other function as a result of aberrant folding of mutant FHL1 proteins. Misfolded protein can result in protein aggregates/reducing body formation, which is associated with more severe disease manifestations in patients with FHL1 mutations. Therefore, mutant mouse models mimicking different FHL1 mutations must be produced in addition to FHL1-null mice to provide further insights into molecular mechanisms relating to FHL1 function and dysfunction.

Compared to FHL1A, there is little research on the minor isoforms FHL1B and FHL1C. Furthermore, studies focusing on the FHL1A isoform have mainly used mouse models or cultured mouse muscle cells. One study, however, specifically investigated the subcellular localization and expression of FHL1B in both healthy and diseased human skeletal muscle [14]. The data from this study was the first to demonstrate a link between FHL1 and the nuclear envelope (NE), as previously the FHL1 isoforms were not believed to be nuclear envelope components. Using confocal immunofluorescent staining of FHL1B, the isoform was detected in the nucleoplasm and at the NE of healthy primary human myoblasts[14]. Additionally, it was observed that FHL1B interacts with both lamin A/C and emerin using *in situ* proximity ligation assay (PLA)[14].

By studying the expression levels of FHL1B in proliferating myoblasts and differentiating myotubes, FHL1B expression was seen to decrease during myoblast differentiation in healthy human myogenic cells [14]. In cells derived from a patient harbouring a FHL

mutation affecting the FHL1A isoform, however, FHL1B was found to be increased (but with normal protein localization), whilst lamin A/C and emerin levels remained unchanged [14]. FHL1B was also found to be upregulated in myoblasts from a LMNA-related EDMD patient[14].

6. SUN1/SUN2

SUN1 and SUN2, along with nesprins-1/2, are major components of the LINC complex and are encoded by *SUN1/SUN2*. SUN1/SUN2 are known to interact with at least four proteins associated with EDMD[112], suggesting that SUN1/SUN2 may be mutated in some individuals with the disease. To test this hypothesis, EDMD patients with no identifiable mutations in EDMD-related genes (*LMNA, EMD, SYNE1/SYNE2*, and *FHL1*) were analysed, and disease-causing variants of *SUN1/SUN2* were identified [11]. Additionally, *SUN1/SUN2* variants were identified in patients with known mutations in causative genes, which appeared to correlate with disease severity [108].

It has been demonstrated that muscular dystrophy-associated mutations in SUN1 or SUN2 impair nuclear coupling to both actin and microtubule networks and disrupt nuclear movement and positioning. In mouse NIH3T3 fibroblasts, most SUN1 and SUN2 variants that were examined inhibited rearward movement of the nucleus [11]. This is a feature that is achieved through LINC complex attachment to actin cables closely associated with the nuclear surface [113], indicating that SUN1 and SUN2 variants may affect the functioning of the LINC complex. Defective nuclear positioning was observed in differentiating myotubes derived from a patient carrying heterozygous *SUN1* mutations [11]. In double SUN1/SUN2 knockout mice, or in mice with nesprin-1/2 disruptions, nuclear positioning in skeletal

muscle is also disrupted leading to nuclear clustering which was also observed in patient myotubes [60,102,111,11].

7. Conclusions

Studies of cell lines, including EDMD patient cells, and mouse models of EDMD, have highlighted muscle cell differentiation and development defects as a shared and prominent feature of EDMD pathology (Table 1). These defects fall broadly into two categories: those that relate to the structural function of the LINC complex, and those that affect protein signalling pathways / gene regulation (Figure 3). What remains to be determined, however, is whether all features of EDMD can be explained by defective structural function of the LINC complex, or whether it is necessary to also invoke defective protein signalling / gene regulation. The focus of this review has been on the role of EDMD-associated nuclear envelope proteins in skeletal muscle, but understanding the expression, localization, and function of these proteins in other tissues may help shed light on this question. It was reported that emerin was present at intercalated discs in the heart [115], for example, offering an explanation for the cardiac conduction defects seen in EDMD, although further work to confirm this may be required [116].

[Figure 3]

At the cellular level, similar differentiation defects are shared between different EDMD-causing mutations, including, for example, problems associated with nuclear positioning resulting from mutations in *LMNA* or *SYNE1*. This review has also highlighted potential differences in terms of the nature of differentiation defects, depending on the mutation in

question. Whilst mutations in *LMNA* or *FHL1* were found to result in premature myogenic differentiation, mutations in *EMD* or *SYNE1* cause delayed differentiation. An important question for future research, therefore, is whether manipulation of *LMNA* and / or *FHL1* (or their protein products) has potential to modify disease pathogenesis caused by mutations in *EMD* or *SYNE1*, and vice versa.

One approach to therapy design for EDMD, perhaps in combination with genetic strategies, may be to target molecules and pathways, downstream of the genetic defect, to help restore cellular homeostasis. Proteomic technologies are well equipped to examine such processes but there is currently a lack of such studies for EDMD research. Researchers have, however, successfully utilised these techniques for identification of biomarkers and novel therapeutic targets in other inherited neuromuscular diseases (for a comprehensive review, see [117]). A notable example is from work in which quantitative proteomics analysis identified perturbed levels of ubiquitin activating enzyme 1 (UBA1) in spinal muscular atrophy (SMA)[118]. Subsequent functional studies demonstrated that restoration of UBA1 is sufficient to ameliorate disease phenotype in mouse and zebrafish models of SMA [119]. In addition to the need for a global (unbiased) view of the EDMD proteome, specific questions also require attention in future. The roles of both *SUN1/SUN2* and *FHL1* in the pathogenesis of EDMD need clarification, for example, as these genes have only recently been linked to the disorder and there is little knowledge of their function in disease. Binding partners of lamin A/C and emerin are implicated in the mechanism(s) of myogenic differentiation and development defects (e.g. Samp1 and Lmo7, respectively), but further work is needed to understand the composition and properties of protein complexes with

which they associate. This may reveal proteins and/or complexes that are able to modulate the expression, activity, or stability of mutant gene/protein in EDMD.

For a long time, embryonic muscle development and regeneration of myofibers were considered as two quite distinctive processes, although it is now generally accepted that they both share common molecular pathways but differ in some aspects of their regulatory processes [120]. A key question in relation to EDMD, therefore, is whether the disease is caused by defective muscle cell differentiation and development pathways or whether defects in shared /overlapping aspects of regeneration pathways are to blame (or both), particularly because clinical symptoms do not appear to be present at birth and develop later in life during adolescence. Longitudinal studies throughout the disease time-course would be invaluable in this respect, if indeed rather challenging due to availability of patient-derived tissues. More broadly, a greater understanding of myogenesis in health and disease may offer insights into the requirements for attaining functional muscle, with a view to recapitulating these processes through pharmacological or genetic interventions in patients with muscular dystrophies including EDMD.

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Figure legends

Figure 1: The LINC Complex. The KASH (Klarsicht/Anc-1/Syne) domain of nesprin-1 giant, found at the ONM, interacts with the SUN (Sad1 and UNC-83) domain of INM SUN1/SUN2 proteins in the perinuclear space, forming a bridge that spans the INM, perinuclear space and INM [18]. Nesprin-1 binds the actin cytoskeleton, whilst nesprin-1α2, a smaller nesprin-1 isoform, directly interacts with kinesin and microtubules [19–21]. Lamin A/C composes the NL that underlies the INM. Emerin is an INM protein that interacts with Samp1, BAF and HDAC3, which also interacts with LAP2β [29,46,50]. FHL1B is an isoform of FHL1 that interacts with emerin and lamin A/C [14]. Image created with BioRender.com.

Figure 2: Nesprin-1 and Nesprin-1α2 Structure. Nesprin-1α2 has a distinctive N-terminal sequence but is otherwise identical to the C-terminal region of nesprin-1 giant [96]. Both contain the highly conserved “Star” region [92], and highly conserved “DV23” exon [92,94]. Figure adapted from [96]. Image created with BioRender.com.

Figure 3: Summary of Conclusions. Three main consequences can arise as a result of mutations in EDMD-associated genes: defective nuclear positioning, premature myogenic differentiation, and / or delayed differentiation. These may have further implications for structural integrity and/or signalling/gene regulation pathways. Currently, the downstream molecular consequences of these problems are uncertain, but may include tissue-specific consequences and implications for muscle regeneration pathways. Image created with BioRender.com.

Figure 1

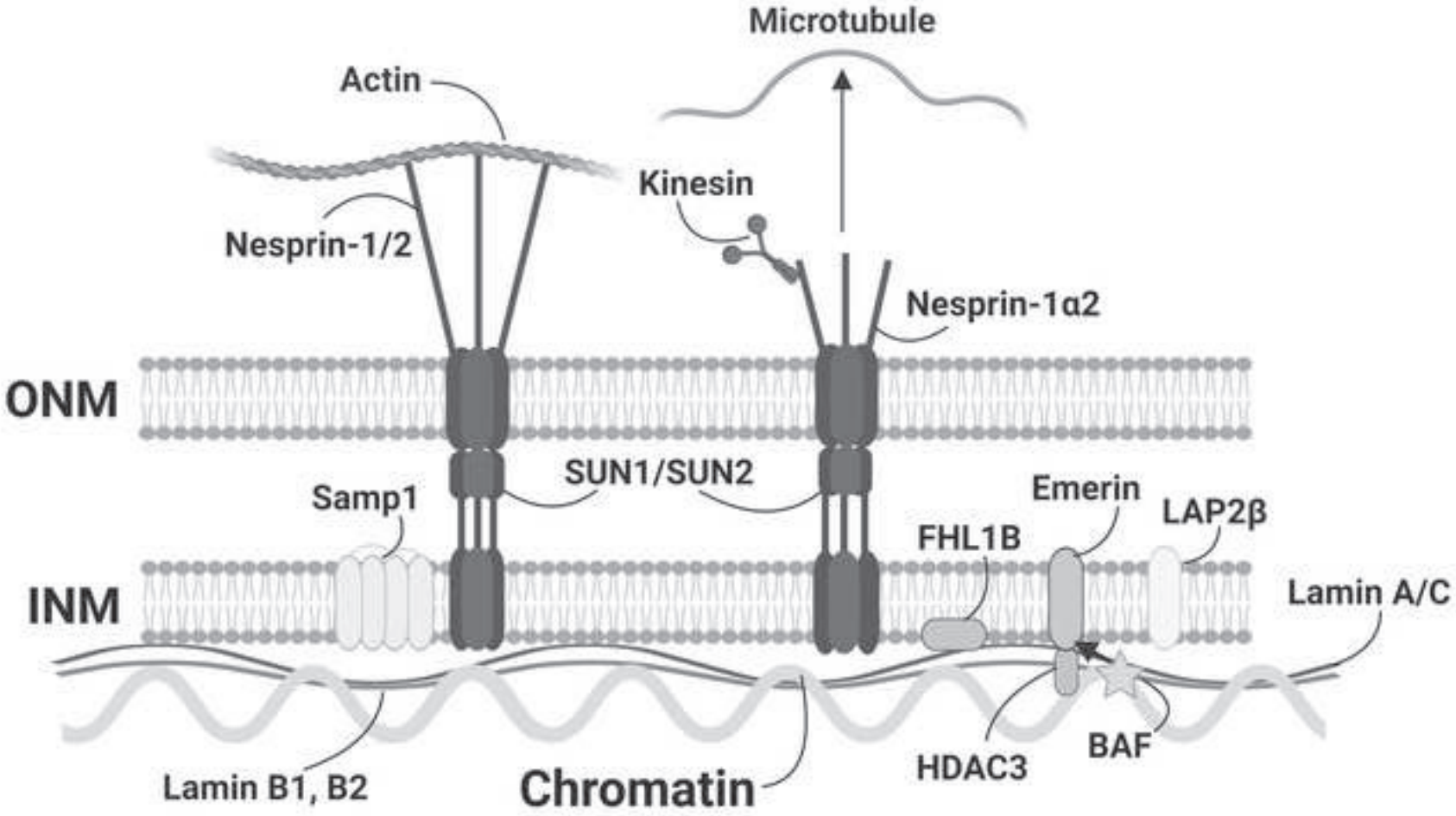

Figure 2

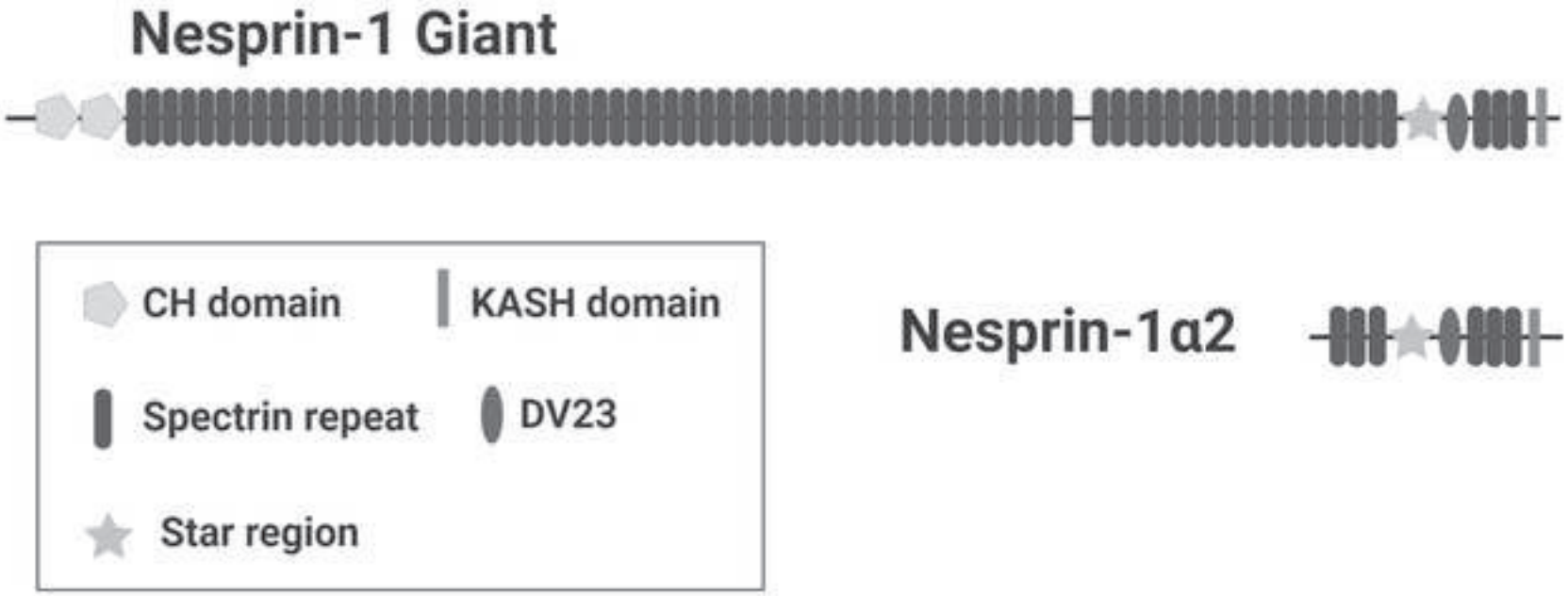

Figure 3

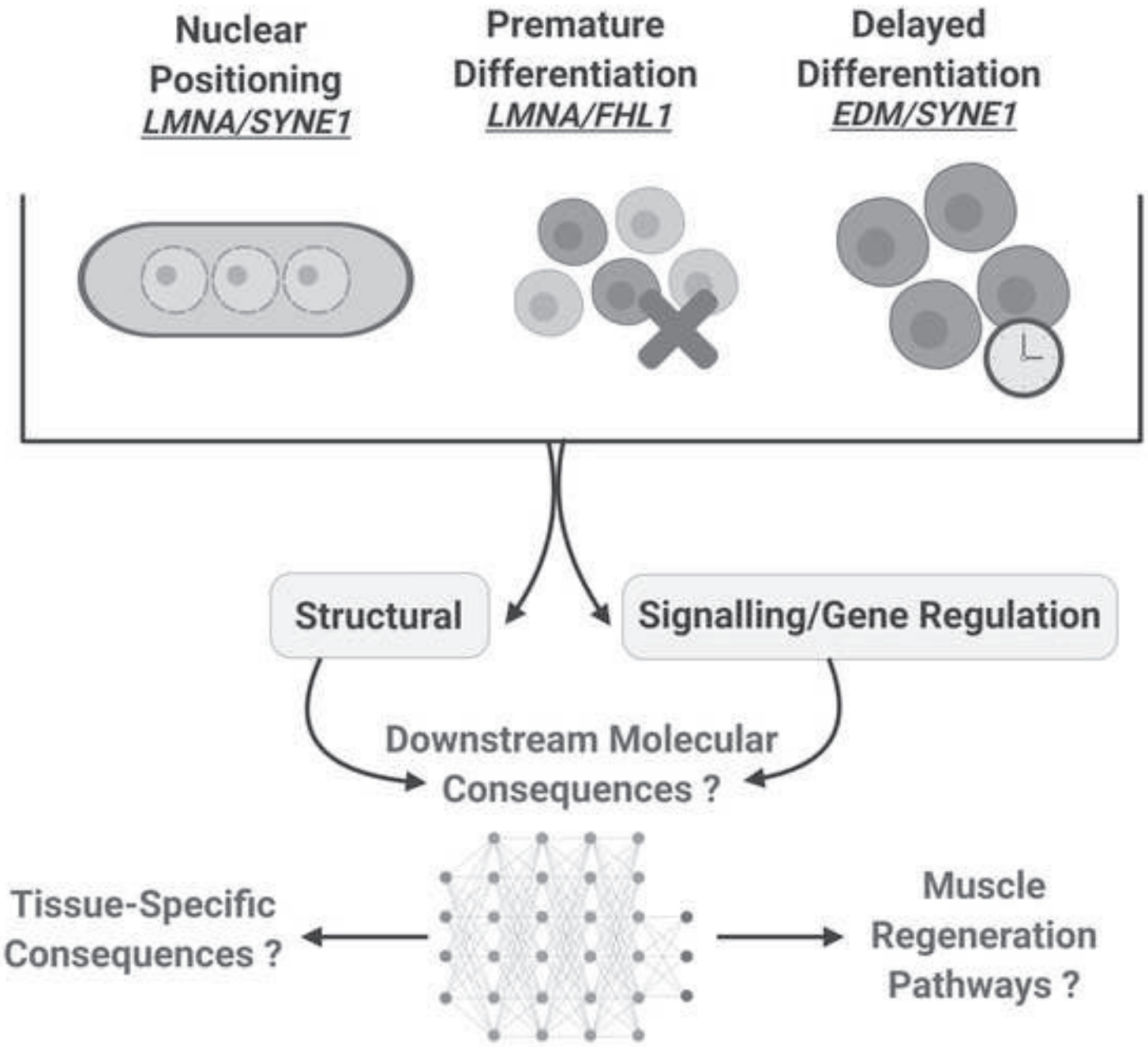

Table (revised)