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SUN proteins and nuclear envelope spacing

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The nuclear envelope consists of 2 membranes separated by 30–50 nm, but how the 2 membranes are evenly spaced has been an open question in the field. Nuclear envelope bridges composed of inner nuclear membrane SUN proteins and outer nuclear membrane KASH proteins have been proposed to set and regulate nuclear envelope spacing. We tested this hypothesis directly by examining nuclear envelope spacing in *Caenorhabditis elegans* animals lacking UNC-84, the sole somatic SUN protein. SUN/KASH bridges are not required to maintain even nuclear envelope spacing in most tissues. However, UNC-84 is required for even spacing in body wall muscle nuclei. Shortening UNC-84 by 300 amino acids did not narrow the nuclear envelope space. While SUN proteins may play a role in maintaining nuclear envelope spacing in cells experiencing forces, our data suggest they are dispensable in most cells.

Keywords: KASH protein, LINC complex, nesprin, nuclear envelope, SUN protein, UNC-84

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Introduction

The nucleus is enclosed by a double membrane structure known as the nuclear envelope. The two membranes of the nuclear envelope, the inner nuclear membrane (INM) and outer nuclear membrane (ONM), are separated by a uniform distance of 30–50 nm.[1] To facilitate the import and export of cargos, the INM and ONM meet at junctions containing nuclear pore complexes (NPCs). Furthermore, the ONM is contiguous with the ER. In addition to its role in containing the genome, the nuclear envelope has been implicated in cell signaling and regulation of many important cellular functions, such as localization of nuclear proteins, organization of heterochromatin, and DNA repair.[2] The role of nuclear envelope defects in a wide-ranging collection of human diseases[2] is drawing increased attention to this complex cellular structure.

In addition to NPCs, connections between the INM and ONM are also formed by linkers of nucleoskeleton and cytoskeleton (LINC) complexes, consisting of Sad1p, UNC-84 (SUN) proteins in the INM interacting with Klarsicht, ANC-1, and Syne homology (KASH) proteins (also known as nesprins) in the perinuclear space (PNS).[3,4] This protein complex is evolutionarily conserved from yeasts to plants and humans. SUN and KASH proteins interact via conserved domains at their C-termini in the PNS.[5] SUN proteins interact with lamins in the nucleoplasm[6,7] and KASH proteins recruit cytoskeletal components to the ONM.[8] In this way, LINC complexes stabilize the nuclear envelope against cytoplasmic forces and facilitate nuclear positioning in a variety of cellular processes, including cell division, establishment of cellular polarity, fertilization, cellular migration, and differentiation. Furthermore, LINC complexes have been hypothesized to maintain the even spacing between the 2 membranes of the nuclear envelope.[9-11]

In *Caenorhabditis elegans* somatic cells, the SUN protein UNC-84 interacts with the KASH protein UNC-83, which recruits microtubule motors kinesin and dynein to facilitate nuclear migration.[12,13] In the early embryo, hypodermal precursors line up in 2 rows across the dorsal midline. As cells intercalate and elongate to form one row of 16 cells, the nuclei migrate contra-laterally to the opposite side of the dorsal midline.[14] The hypodermal cells eventually fuse, and in the resulting syncytia, hypodermal nuclei are

anchored evenly throughout. In *unc-84* or *unc-83* mutant embryos, the elongation and intercalation of cells proceeds normally, but the nuclei fail to move from their initial positions.[13,15] As the embryo continues to develop, mutant nuclei that fail to migrate are passively pushed toward the dorsal midline by underlying muscle cell migrations and can be observed by DIC microscopy in the dorsal cord of the L1 larva.[15] In the adult hypodermal syncytia, UNC-84 interacts with ANC-1, which interacts with actin to anchor nuclei .[16] After cell fusion, in *unc-84* or *anc-1* mutants, the nuclei are unanchored and are free to drift throughout the syncytia, often clustering in groups.[16,17]

SUN Proteins and Nuclear Envelope Spacing

Most SUN proteins are large, with more than half of the protein residing in the PNS. The C-terminal ~200 amino acids contain the conserved SUN domain, which trimerizes into a cloverleaf structure with the N-terminal stalk formed by a right-handed trimer coiled-coil.[5,18] While the structure of the linker domain between the transmembrane domain and the trimeric coil is unknown, it has been proposed that the trimeric coiled-coil continuously extends to the transmembrane domain, forming a rod approximately 45 nm long. Such an extended coil structure would be suitable to span the space between the ONM and the INM and serve as a "molecular ruler" to regulate even spacing of the nuclear envelope[10,11] (**Fig. 1A**). Interestingly, several divergent SUN proteins, including human SUN3-5,[19-21] which are restricted to the testis, and *C. elegans* SUN-1,[22,23] which is restricted to the germ line, are predicted to have much smaller luminal domains than those of their somatic counterparts. The prediction is that tissues expressing these SUN proteins would display narrower nuclear envelope spacing (**Fig. 1B**), but nuclear envelope spacing in those tissues has not been directly examined.

The hypothesis that LINC complexes may serve as molecular rulers for nuclear envelope spacing was primarily based on electron micrographs of HeLa cells either depleted for SUN1 and SUN2 or expressing a soluble dominant-negative SUN domain fragment, showing large distortions of the PNS and ~100 nm separation of the ONM away from the INM[9] (**Fig. 1C**). However, HeLa cells in culture are in a very different environment than most nuclei *in vivo*. Because they make extracellular contacts to a hard surface, the cytoskeleton is under increased strain. Stresses at the cell surface can cause long-range force propagation, extending to the nucleus and beyond.[24] As a result, nuclei in cell culture are flattened, like a pancake.[25] In live tissue, nuclei are usually more spherical and are presumably not subject to the same intracellular forces.

The *C. elegans* system is particularly suited to address this question directly. UNC-84 is the only SUN protein expressed in somatic tissue, yet null mutants are viable and fertile. In *unc-84*

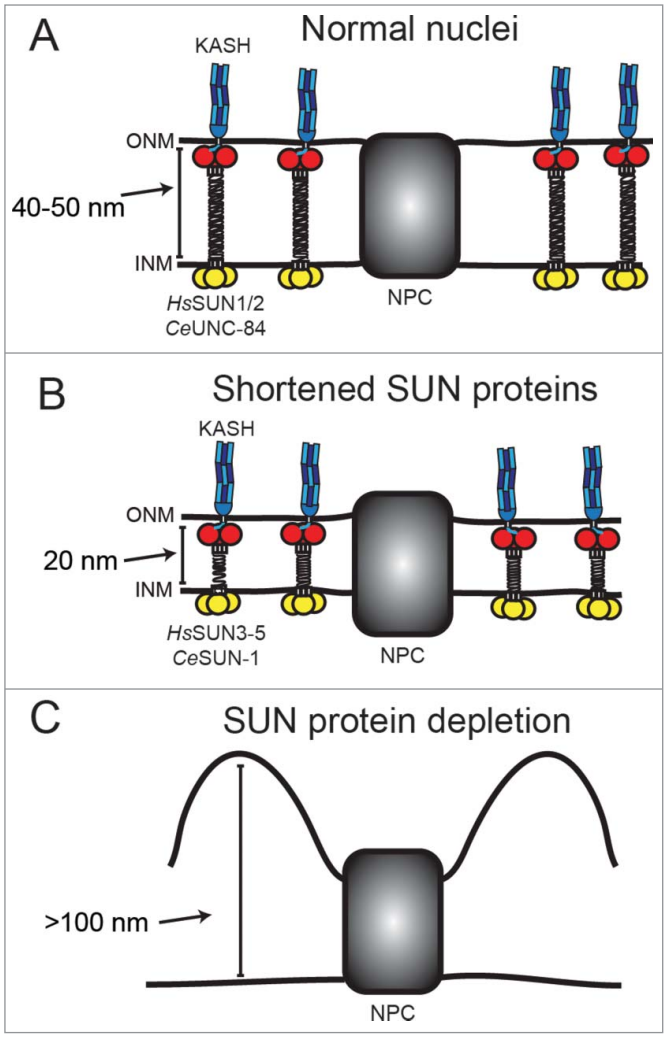

Figure 1. SUN proteins are predicted to regulate nuclear envelope spacing. In this figure, SUN proteins are depicted in the inner nuclear membrane (INM) with their nucleoplasmic domain in yellow and their conserved SUN domain in red. SUN domains bind KASH proteins (blue) in the outer nuclear membrane (ONM). NPC are nuclear pore complexes where the inner and outer nuclear membranes are connected. (**A**) The linker domains of human SUN1/2 and *C. elegans* UNC-84, between the trans-membrane span and the SUN domain, are predicted to form trimeric rods that span the 40–50 nm distance between the inner and outer nuclear membranes. (**B**) Shorter SUN proteins (human SUN3–5 and *C. elegans* SUN-1) are predicted to have shorter luminal domains and, as a result, narrower nuclear envelope spaces. (**C**) In the absence of LINC complexes, lack of connection of the cytoskeleton to the nucleoskeleton is expected to cause the ONM to separate from the INM.[9]

(null) tissues, both UNC-83 and ANC-1 fail to localize, which allowed us to examine nuclei completely lacking LINC complexes.[12,16] High pressure freezing and electron microscopy techniques are well established in *C. elegans*,[26] which allowed us to expand our observations to a variety of tissues and developmental stages. The defects of the *unc-84(null)* mutant can be fully rescued by an UNC-84 transgene,[12] which allowed us to express truncated forms of UNC-84 as the sole somatic SUN protein in an otherwise null background and to examine their effects on nuclear migration. We previously used this technique to locate the transmembrane domain in UNC-84, as well as multiple sorting motifs in the N-terminus required for INM localization.[27]

UNC-84 Maintains the NE Spacing in Force-bearing Cells but is Dispensable in other Tissues

The absence of LINC complexes caused significant deformations of the nuclear envelope in HeLa cell culture, but whether a similar phenotype would be observed in an animal lacking LINC complexes remained an open question. The hypothesis that LINC complexes serve as molecular rulers suggests that a SUN-less animal would display gross nuclear envelope defects in most or all tissues throughout development. We therefore carried out a series of experiments to characterize the role of the SUN protein UNC-84 in the architecture of the nuclear envelope.[28] We began by examining pre-morphogenesis embryos, near the stage where UNC-84 and UNC-83 function together to move nuclei contra-laterally across the dorsal midline of the developing hypodermis. In most wild type nuclei, the PNS is consistently 30–50 nm in width, in agreement with previously published results.[29] Surprisingly, nuclei from the null mutant *unc-84(n369)* strain are not significantly different than the N2 wild type laboratory strain. The PNS of most *unc-84* mutant nuclei are evenly spaced throughout, and are not significantly wider than wild type. Similarly, in the first larval stage, most nuclei, including those of the pharynx and hypodermis, have normal nuclear envelope spacing in both wild type and *unc-84 (n369)* animals (**Fig. 2A**). Therefore, in contrast to HeLa cells, where an absence of LINC complexes results in extreme separation of the ONM away from the INM, in most *C. elegans* tissues, removal of LINC complexes has little to no effect on nuclear envelope spacing. Cells in the interior of the animal likely have less rigid cell-cell contacts than tissue culture cells, resulting in less overall strain to the cytoskeleton.

In the *C. elegans* larva, the best candidates for a cell type with increased intracellular tension resulting from cellular shape changes are striated body wall muscles. In contrast to most other tissues where nuclei are nearly spherical, muscle nuclei are oblong, with the long axis oriented parallel to the muscle fibers.[30] The PNS of muscle nuclei are an even 40–50 nm width all around the nuclei, although in some nuclei, the PNS narrowed along the long axis and/or widened at the ends. We then examined *unc-84 (n369)* muscle nuclei to determine if LINC complexes play a role in stabilizing the nuclear envelope space in regions where forces appear to be highest (**Fig. 2B**). Indeed, *unc-84* mutant muscle nuclei have PNS widths of 100–500 nm. As with wild type nuclei, the largest distortions are at the ends of nuclei, although on occasion, smaller blebs are observed on the long axes (arrow in **Fig. 2C**). Each nucleus was observed over multiple serial sections, which allowed for observation of the change in shape of the blebs section by section. In the z-plane, the blebs start out small, grow larger near the centers of nuclei, and are imperceptible at the bottom surfaces of nuclei. Therefore, body wall muscle is the only *C. elegans* tissue of the wide variety of embryonic and larval tissues we observed that produces large nuclear envelope distortions similar to those observed in HeLa cells.[9]

The observation that most nuclei in the *unc-84(n369)* display normal nuclear envelope spacing does not in and of itself contradict the hypothesis that LINC complexes set the dimensions of the PNS. Because UNC-84 is required to recruit UNC-83 to the ONM, *unc-84(n369)* nuclei do not have UNC-83 at the ONM and therefore lack a direct connection to motors that pull on the nuclear envelope in wild type nuclei. A direct test of this

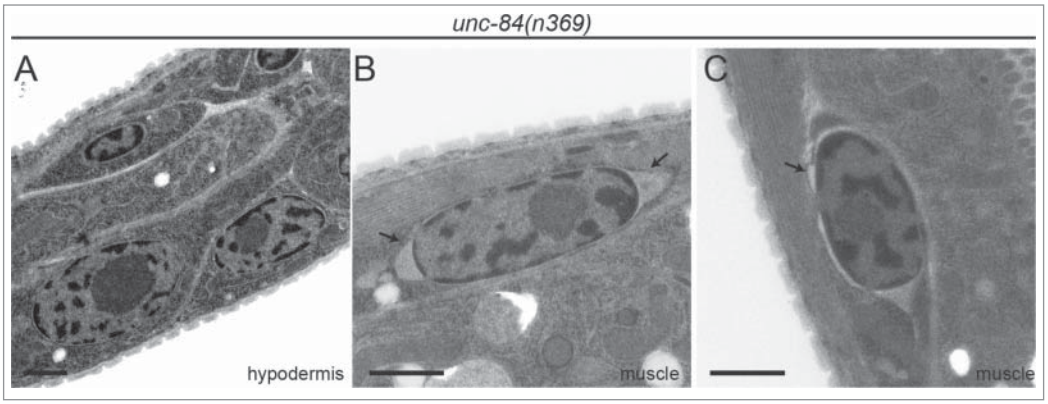

Figure 2. UNC-84 is required for even nuclear envelope spacing only in body wall muscle cells. (**A**) Hypodermal larval nuclei in the *unc-84(n369)* animal do not display blebbing of the nuclear envelope. (**B-C**) However, large distortions of the nuclear envelope were observed at the ends of body wall muscle nuclei (arrows in B) and occasionally, along the sides (arrow in C). Scale bars are 1μm.

hypothesis required the introduction of an abnormally sized SUN protein that retains the ability to recruit and interact with KASH proteins. The hypothesis predicts that the PNS should then change to accommodate this mutant SUN protein in all tissues where it is expressed. The majority of the linker domain of UNC-84 in the PNS between the trans-membrane span and the conserved SUN domain bears little sequence similarity to other SUN proteins. Hydropathy analysis revealed multiple regions of increased hydrophobicity in the linker domain of UNC-84. Additionally, a hybrid protein with the *C. elegans* UNC-84 nucleoplasmic domain fused to the human SUN1 luminal domain, which contains 2 predicted coiled-coil domains,[31] functioned normally in hyp7 nuclear migration, suggesting the linker domain is functionally conserved from worms to mammals. These two findings are consistent with a model where the linker domain of UNC-84 assumes a helical structure. Also consistent with this model is our finding that deletion of 15 amino acids immediately preceding the SUN domain of UNC-84, which correspond to the 15 amino acids of human SUN2 that form the coiled stalk of the trimer, completely disrupts recruitment of UNC-83. Indirectly, this also suggests that UNC-84 functions as an oligomer *in vivo*. Intriguingly, deletion of approximately 300 amino acids of the UNC-84 linker domain (residues 556–861 between the transmembrane span and the conserved SUN domain; **Fig. 3A**) nonetheless produced a functional UNC-84 protein that was able to recruit UNC-83 to the ONM and move nuclei. This UNC-84 truncation therefore provided the perfect tool to directly test the hypothesis that SUN proteins set nuclear envelope spacing. The nuclear envelope spacing in embryos expressing *unc-84 (Δ556–861)* is not noticeably narrower than wild type. Nor is there significant narrowing of PNS width in the muscle nuclei of larvae.

Our results suggest that rather than setting nuclear envelope spacing, SUN proteins have evolved to span the distance between the INM and ONM. The question remains: if SUN proteins do not determine nuclear envelope spacing, what does? NPCs certainly play a role, as the perinuclear space narrows around NPCs in nuclei without LINC complexes. However, our results,[28] as well as SUN protein knockdown in tissue culture,[9] indicate that NPCs are not sufficient to maintain 40–50 nm spacing away from the pores. The answer may lie in the nuclear envelope's closest relative – the ER. The membranes of the nuclear envelope share many properties with the ER and the shape of the nuclear envelope echoes the shape of ER. During interphase, ER membranes are mostly in sheets, and polyribosomes are thought to provide the force to keep them flat.[32] ER sheets also have a characteristic spacing, although it is observed to be approximately twice as large as that of the perinuclear space in mammalian cells.[33] The morphology of ER sheets is maintained by the luminal spacer Climp63; overexpression of Climp63 results in an increase of ER sheets when overexpressed. Intriguingly, and in agreement with our results, depletion of Climp63 does not, in fact, result in blebbing of ER membranes. Rather, the intermembrane distance in the ER is reduced by half and more closely approximates the nuclear envelope distance, which is unaffected by Climp63 levels.[33] This suggests

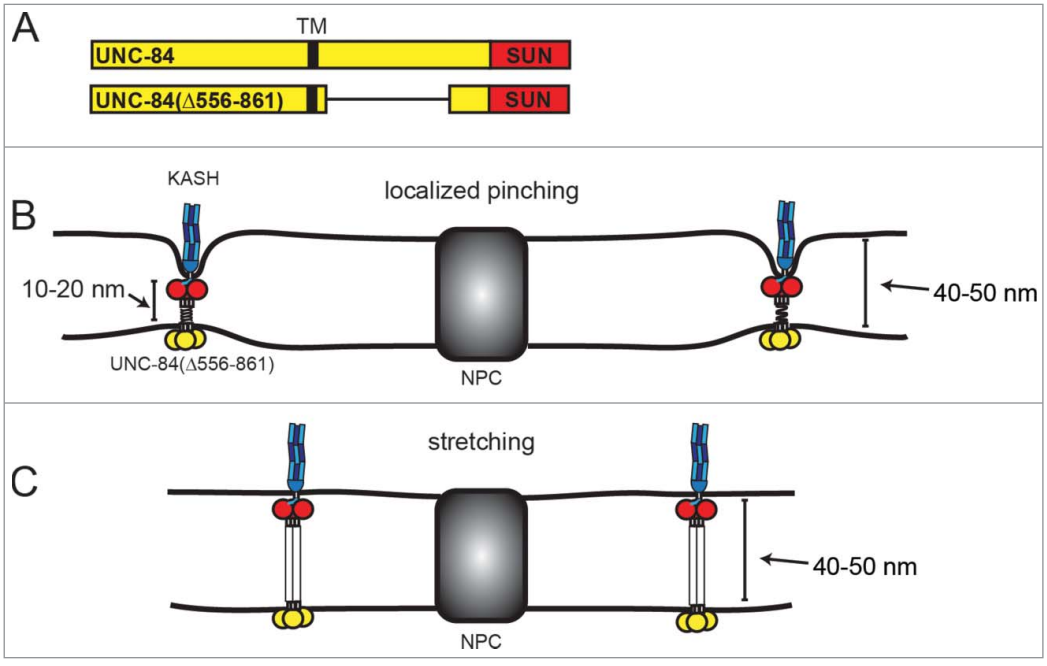

Figure 3. Two hypotheses for how UNC-84(Δ556–861) interacts with KASH proteins without narrowing the perinuclear space. SUN and KASH proteins are depicted as in **Figure 1**. (**A**) Schematics of full-length UNC-84 and the functional truncation mutant UNC-84(Δ556–861). The transmembrane domain (TM) is depicted in black. (**B**) LINC complexes may be spaced far apart and localized pinching together of the 2 membranes may not be resolvable by TEM. (**C**) The luminal domain of UNC-84(Δ556–861) may be fully extended to accommodate the normal 40–50 nm perinuclear space.

that Climp63 functions to expand the width of ER sheets to make them bigger than the default state found in the nuclear envelope. As the ONM is contiguous with the ER, polyribosomes could similarly be maintaining its flatness in the absence of LINC complexes. Chromatin could provide a similar force on the nucleoplasmic side of the nuclear envelope. In most tissues, these forces may be stronger than forces applied on the nucleus by the rest of the cell, such that even in the absence of SUN proteins, the nature of the membranes is to lay flat and evenly spaced. Therefore, the even spacing of the nuclear envelope in the absence of LINC complexes may be similar to the default spacing for ER sheets lacking Climp63. Alternatively, there may be other inner nuclear membrane proteins with large luminal domains that mediate perinuclear spacing. By contrast, body wall muscle cells, or cells adhered to a dish, experience more mechanical strain than most *in vivo* cells. These forces are presumably strong enough to overcome the forces working to keep the nuclear envelope flat in the absence of LINC complexes.

Another question is if the distance between the ONM and INM is an inherent property of the membranes, how can UNC-84(Δ556–861) mutant interact with KASH proteins without narrowing the PNS? Membranes could be pinched inward in areas too small to be resolved by EM (**Fig. 3B**). Little is known about the distribution of LINC complexes along the nuclear envelope. Very few SUN/KASH interactions might be required to stabilize the nuclear envelope. If only a few, sparse LINC complexes are required to move a nucleus (**Fig. 3B**), the pinching that results might not be resolvable under the sectioning and electron microscopy conditions we used. Alternatively, the remaining 60–80 residues could be fully stretched out. While the full-length UNC-84 protein may contain a trimeric helical rod that spans the distance between the ONM and INM, the UNC-84(Δ556–861) mutant might not assume the same conformation. It is possible that instead of a rod, each linker region is an unordered polypeptide chain, with a greater capacity to extend than the full-length version (**Fig. 3C**). If each residue could fully extend to 3.8 Å in an open peptide backbone,[34] the remaining residues in the linker domain could reach 20–25 nm. Adding on an additional 14–15 nm (5 each for the ONM and INM and 4–5 for the SUN domain), gives a total of ~40 nm, similar to the distances observed in nuclei expressing UNC-84(Δ556–861).

Nuclear Envelope Morphology and Disease

The observation that the absence of LINC complexes causes nuclear envelope architecture defects in cells under strain could have implications for understanding some human diseases. In *C. elegans*, nuclear envelope defects were only observed in striated body wall muscle cells. Interestingly, the largest group of laminopathies consists of those affecting striated muscle tissues.[2] One such disease, Emery-Dreifuss Muscular Dystrophy (EDMD), is associated with mutations in several nuclear envelope proteins, including Nesprin-1 and -2 and SUN1.[35,36] EDMD patients display early tightening of the elbows, Achilles tendons, and neck, followed by progressive muscle wasting in the limbs and associated dilated cardiomyopathy.[37] Mutations in nesprin-1 have also been linked to dilated cardiomyopathy without skeletal muscle defects.[38] The mechanism by which these mutations cause disease in a tissue-specific manner with variations from patient to patient is poorly understood. Our results suggest a possible involvement of defects in nuclear envelope structure. We compared the swimming motility of *unc-84(n369)* larvae, near the same stage where nuclear envelope defects were observed by TEM, to wild type control animals. Using both manual and computational scoring techniques the *unc-84(n369)* animals have an irregular swimming pattern compared to wild type. Mutant animals often coil for extended periods and have extra twitches. Notably, the motility defects we observed cannot be attributed to the loss of ventral cord neurons that has been previously described,[15] as the required nuclear migration event in precursor (P) cells occurs at a later stage in development. Thus, we have identified a novel locomotion defect in the *unc-84(n369)* mutant consistent with a muscle contraction defect. As the structure of the muscle fibers in the mutant animal were not distinctly different from wild type, the nuclear envelope architecture defects could contribute in some way to the locomotion defects in live animals. However, a neuronal defect in a different cell lineage could lead to the locomotion disorder. A potential candidate is the lumbar ganglion neuron PVQ, which is mispositioned in 15% of *unc-84(n369)* animals.[39] Nonetheless, our results open a new area for exploration in better understanding the relationship between muscle disease and nuclear envelope architecture.

Conclusion

Our work has clarified the prevailing model for the role of SUN proteins in nuclear envelope spacing. SUN proteins are necessary to maintain the even spacing between the INM and ONM in cells under high mechanical strain, but are not needed in other cells, where the inherent characteristics of ER-derived membranes are sufficient to keep them flat and evenly spaced. We also have also uncovered a potential functional consequence of nuclear envelope architecture defects in muscle tissue. Further experiments are needed to better understand the nature of this connection and the role it might play in human disease. Specifically, examination of nuclear envelope ultrastructure in tissues from mouse muscular dystrophy models, as well as patient samples, should be informative.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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