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Building and breaking mechanical bridges between the nucleus and cytoskeleton: Regulation of LINC complex assembly and disassembly

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Abstract

The nucleus is physically coupled to the cytoskeleton through LINC complexes, macromolecular bridges composed of SUN and KASH proteins that span the nuclear envelope. LINC complexes are involved in a wide variety of critical cellular processes. For these processes to occur, cells regulate the composition, assembly, and disassembly of LINC complexes. Here we discuss recent studies on the regulation of the SUN-KASH interaction that forms the core of the LINC complex. These new findings encompass the stages of LINC complex assembly, from the formation of SUN-KASH heterooligomers to higher-order assemblies of LINC complexes. There is also new work on how components of the LINC complex are selectively dismantled, particularly by proteasomal degradation. It is becoming increasingly clear that LINC complexes are subject to multiple layers of regulation.

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Introduction

The linker of nucleoskeleton and cytoskeleton (LINC) complex is an evolutionarily conserved nuclear envelope-spanning macromolecular bridge that physically connects chromatin and the nuclear lamina to the cytoskeleton [1,2]. The LINC complex is composed of two types of type-II membrane proteins: Sad1/UNC-84 (SUN) proteins located in the inner nuclear membrane (INM), and Klarischt/ANC-1/SYNE homology (KASH) proteins that are embedded in the outer nuclear membrane (ONM) [3]. KASH proteins contain a conserved C-terminal transmembrane domain followed by a short ($\sim$10–32 residue), conserved luminal KASH peptide. Within the cytoplasm, KASH proteins interact with various cytoskeletal elements and signaling molecules [4,5]. KASH proteins are targeted to the nuclear envelope via a direct physical interaction between their KASH peptide and the conserved C-terminal SUN domain of SUN proteins in the perinuclear space of the nuclear envelope [3,6–8]. The KASH peptide is both necessary and sufficient to target proteins to the ONM [9]. Divergent SUN protein C-termini extend into the nucleoplasm, where they interact with lamins, chromatin-binding proteins, and other proteins [10]. The KASH-SUN interaction allows the LINC complex to transmit mechanical forces from the cytoplasm across the nuclear envelope and into the nucleoplasm. Consequently, LINC complexes are necessary for several fundamental cellular processes, including DNA damage repair, meiotic chromosome pairing, nuclear positioning, force transmission across the nuclear envelope, and the mechanoregulation of gene expression [10,11]. Mutations in KASH and SUN proteins are also associated with a variety of diseases [12,13*]. Regulating how, when, and where LINC complexes assemble and disassemble is therefore a fundamental task that the cell must perform to carry out a wide variety of cellular processes.

The LINC complex is not a singular complex — it is a modular class of complexes that consists of different combinations of SUN and KASH proteins. Mammals possess two ubiquitously expressed SUN proteins (SUN1 and SUN2) as well as three testes-specific SUN proteins (SUN3, SUN4, and SUN5) [10]. Mammals also have six KASH proteins: nuclear envelope spectrin-enriched protein (nesprin)-1, −2, −3, −4, KASH5 (a.k.a. CCDC155), and KASH6 (a.k.a. Jaw1/LRMP) [10,14]. It is currently unknown if and how the modularity of the LINC complex may contribute to its assembly and/or disassembly. For example, a variety of KASH proteins can bind to a single homotrimer of SUN1 or SUN2 [15,16]. There is also evidence that SUN1 and SUN2 can form heterotrimers [16,17*–20]. The two widely expressed SUN proteins and six KASH proteins allow for hundreds of possible unique LINC complexes. Given this large "state space" that LINC complexes can exist within, how do cells regulate LINC complex

assembly and disassembly? Recent work has focused on the assembly of the core SUN-KASH interaction, the variety of structural configurations that the various SUN-KASH combinations can form, and the mechanisms cells use to selectively degrade particular SUN or KASH proteins. These mechanisms are summarized below and in Figures 1 and 2.

SUN protein homotrimerization

The process of LINC complex assembly begins with SUN protein homotrimerization [21]. One model is that SUN protein monomers adopt an autoinhibited state that prevents homotrimerization as they are being transported from the endoplasmic reticulum to the INM [22,23] (Figure 1b). This may be mediated by

Figure 1

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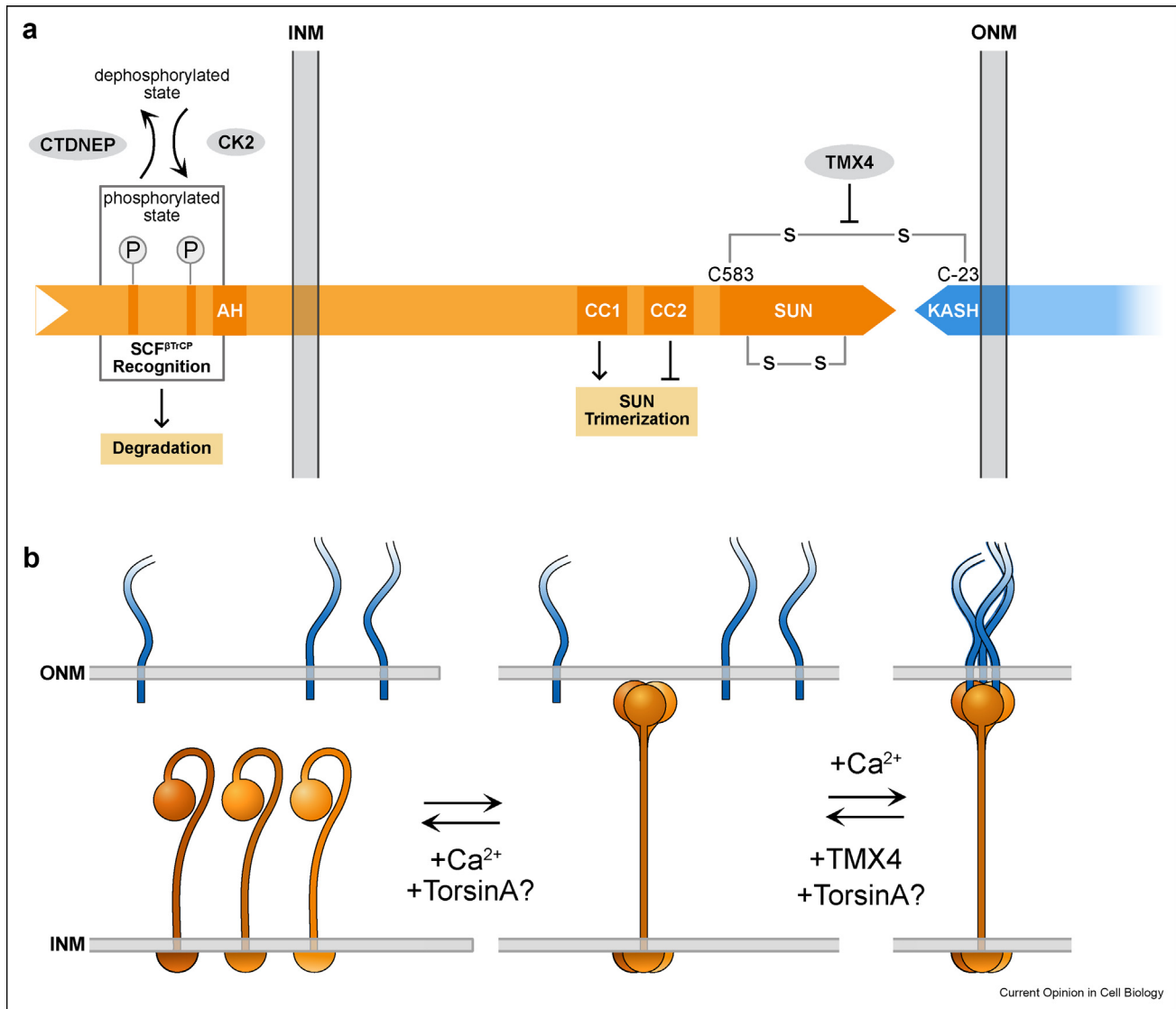
Points of regulation for LINC complex assembly and disassembly (**a**) Diagram of the SUN-KASH interaction's points of regulation. The domains of human SUN2 are in orange, and the KASH peptide is in blue. Proteins are drawn as arrows pointing from the N- to the C-terminus. The inner nuclear membrane (INM) and the outer nuclear membrane (ONM) are drawn as grey bars. The nucleoplasmic portion of SUN2 contains regulatory sites for proteasomal degradation, including two phosphorylation sites and an amphipathic helix (AH). The luminal portion of SUN2 contains coiled-coil regions (CC1 and CC2) that are important for the regulation of SUN trimerization. The SUN-KASH interaction includes an inter-molecular disulfide bond between SUN2's C583 and the KASH peptide's cysteine at position −23 (23 amino acids from the C-terminus). (**b**) Model of LINC complex assembly. The SUN domains (orange spheres) can be in an autoinhibited state by interacting with the rest of its luminal domain (orange stalk). The second step of LINC assembly is trimerization of the SUN proteins. The core of LINC complex assembly is complete with the KASH peptides bind to the SUN trimers. In some contexts, increased levels of calcium lead to decreased trimerization of SUN proteins. TorsinA is thought to break apart LINC complexes, though the stage it acts upon is unknown. TMX4 breaks the inter-molecular disulfide bond between SUN and KASH, weakening their interaction.
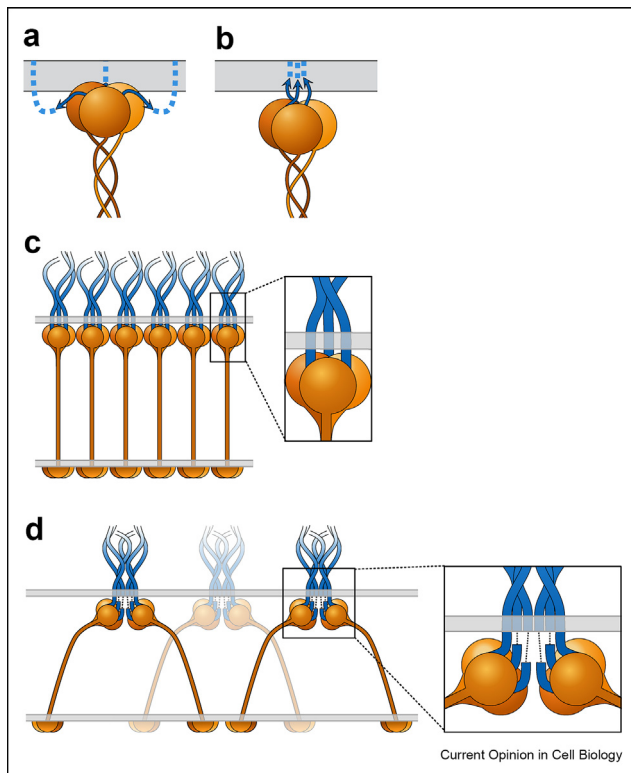
LINC complex binding modes and higher-order assembly models (**a**) Binding mode 1. Here the N-terminus of the KASH peptide turns downwards (towards the SUN N-terminus) as it extends past the binding interface. (**b**) Binding mode 2. The N-termini of these KASH proteins do not make a turn, and instead extend up towards the outer nuclear membrane. (**c**) Higher-order LINC assembly. In this model, multiple LINC complexes associate laterally. This configuration of LINC complexes allows the KASH peptide to reach its binding interface in the SUN trimer while still being inserted into the INM (inset). (**d**) Head-to-head model. In this model the SUN trimers of neighboring LINC complexes face each other and bind in a "head-to-head" orientation. This SUN-to-SUN inter-action as well as the binding of three KASH peptides to each SUN trimer forms a hetero-hexamer. With the SUN trimer angled away from the ONM, the KASH peptides are likely too short to both bind to the SUN domain and to have their transmembrane domains inserted into the ONM. This discrepancy in KASH peptide lengths is shown as dashed lines (inset).

interactions between a portion of the SUN domain that, when active, clamps down on the KASH peptide called the KASH lid, and an immediately N-terminal coiled-coil (CC)-containing region known as the auto-inhibitory domain (AID) [22,24]. SUN1 and SUN2 both possess two conventional CC-containing regions, CC1 and CC2, within their luminal domains (Figure 1a). In SUN2, the AID is a non-conventional CC, whereas the AID of SUN1 overlaps with CC2. The AID-SUN domain interaction locks SUN proteins in an inactive conformation that cannot homotrimerize or interact with KASH peptides. In contrast, CC1 is proposed to promote SUN protein homotrimerization [22,25]. While it is not known exactly how SUN proteins transition

from monomeric to homotrimeric states, the homo-trimerization process could be influenced by changes in the concentration of calcium ions within the perinuclear space; lower concentrations of calcium favor SUN pro-tein homotrimerization at pH 8.0[25].

Interestingly, another report demonstrated that *in vitro* SUN-KASH interactions were promoted by the addition of physiological levels of calcium [17]. How can calcium both inhibit LINC complex assembly by decreasing homotrimerization and promote assembly by increasing SUN-KASH interactions? One potential explanation for these differing results may stem from the use of the isolated luminal domain of SUN2 vs. a full-length SUN2 protein reconstituted in a supported lipid bilayer.

SUN-KASH interactions: intermolecular SUN-KASH disulfide bonds
SUN-KASH interactions often include an intermolec-ular disulfide bond between conserved cysteines in the SUN domain and in the KASH peptide (Figure 1a) [21]. Molecular dynamics simulations show that this covalent linkage enhances the LINC complex's ability to trans-mit forces [26,27]. In addition, this intermolecular di-sulfide bond is necessary for LINC complex-dependent processes, including ANC-1 dependent nuclear anchorage in the developing *C. elegans* hypodermis and rearward nuclear movement during centrosome orien-tation in fibroblasts polarizing for directional cell migration [27]. A mutation that prevents SUN2 from forming its intermolecular disulfide bond with KASH peptides reduces the levels of SUN2 protein in the cell [28*] — suggesting that regulation of this disulfide bond may regulate the overall stability of LINC complexes.

Thioredoxin related transmembrane protein 4 (TMX4) is a regulator of the SUN-KASH intermolecular disulfide bond. TMX4 is a protein disulfide isomerase (PDI), a family of over 20 proteins that modify disulfide bonds and largely reside in the endoplasmic reticulum [29]. TMX4 is one of a few PDI proteins that is enriched in the nuclear envelope [30**,31]. Nesprins are the clients of TMX4: TMX4 breaks the intermolecular SUN-KASH disulfide bond and forms a mixed disulfide with the nesprin [30**]. Further investigation of TMX4 function within the nuclear envelope will reveal if it plays a regulatory role in LINC complex-dependent processes or if it affects LINC complex stability. Future studies should investigate the potential roles of the remaining members of the PDI protein family during LINC com-plex regulation.

SUN-KASH interactions: binding modes
The next step after trimerization in LINC complex assembly is the SUN protein's SUN domain binding to the KASH protein's KASH peptide within the peri-nuclear space of the nuclear envelope (Figure 1b). The SUN-KASH binding interface that forms the core of

the LINC complex was recently investigated in detail by several structural studies. The crystal structures of human SUN1 and SUN2 bound to the KASH peptides of human nesprin1-4 and KASH5 have been reported [15,21,32,33]. These structures allow us to more precisely identify the binding interactions that are most likely important for establishing the LINC complex-mediated mechanical linkage across the nuclear envelope.

The above-described work shows that different KASH peptides bind to SUN homotrimers in two distinct modes (Figure 2a and b). The C-terminal ten residues of KASH peptides bind within common interfaces formed between neighboring SUN protomers in a SUN protein homotrimer and under the KASH lids of SUN domains [15]. However, the way that the KASH peptide exits this binding pocket varies. Nesprin-1 and -2 bind SUN2 such that their KASH peptides make a 90° turn at a conserved proline at position −11 residues from the C terminus and continues out towards the periphery of the SUN2 homotrimer [15] (Figure 2a). This positions the KASH peptide so that the conserved −23 cysteine can form an intermolecular disulfide bond with SUN2's conserved C583 [21]. Nesprin-1 binds to SUN1 homotrimers in a similar configuration [32].

In contrast, crystal structures suggest that nesprin-3, -4, and KASH5 bind to SUN2 homotrimers such that their KASH peptides extend up towards the KASH lid of the neighboring SUN protomer [15] (Figure 2b). Nesprin-4 and KASH5 binding to SUN1 homotrimers display a similar configuration [32]. Nesprin-3, -4, and KASH5 have a leucine instead of a proline at position −11, allowing the rest of the KASH peptide to extend in a relatively straight configuration away from the SUN domain interface [15].

These results show that the different KASH peptides exhibit two distinct binding modes: the outward turn of nesprin-1 and -2 bound to SUN homotrimers, or the upward extension of nesprin-3, -4, and KASH5 bound to SUN homotrimers. One thing not explained by these structures is that the KASH peptide of nesprin-3 has a conserved −23 cysteine like nesprin-1 and nesprin-2, but it adopts the same binding mode as nesprin-4 and KASH5 [15]. Therefore, it is unclear how the −23 cysteine of nesprin-3 is positioned to form a disulfide bond with SUN2. It is possible that the crystal structures are capturing just a subset of a few possible different binding modes for each SUN-KASH pair.

It is currently unknown how these different binding modes influence the interaction of the LINC complex with the ONM. The length of the KASH peptide that protrudes out of the ONM and into the perinuclear space is relatively short, only 10−32 residues depending on the KASH protein [34]. For the longer KASH peptides, with conserved cysteines at position −23, there are 6−8 residues extending beyond the interaction with the SUN domain. These residues are usually acidic and are not captured in any crystal structures, but in molecular dynamic simulations, they can easily reach the ONM [35]. For the shorter KASH peptides, like KASH5 and *C. elegans* UNC-83, the linker is not long enough to reach the ONM after exiting the KASH lid of the SUN homotrimer without forcing the conserved hydrophobic patch present on outer surface of the KASH lid of the SUN domain into the lipid bilayer (Figure 2a and b) [35]. Further structural studies performed on full-length KASH and SUN proteins reconstituted in supported lipid bilayers are needed to understand how these different binding modes may affect LINC complex assembly.

Higher-order LINC complex assemblies

How, when, and why do multiple LINC complexes interact with each other? Higher-order LINC complex assemblies are known to form in cells and are necessary for performing biological functions. In fibroblasts polarizing for migration, SUN2 and nesprin-2G form linear arrays along dorsal perinuclear actin cables called transmembrane actin-associated nuclear (TAN) lines — this is necessary for rearward nuclear movement to occur [36,37]. SUN2 has also been found to form clusters as a part of the endoplasmic reticulum stress response [38]. In addition, higher-order SUN1-containing LINC complexes can form rings around nuclear envelope-associated meiotic telomeres, which are involved in meiotic chromosome pairing during meiotic prophase [39].

How do LINC complexes form higher-order assemblies, and how are the SUN and KASH proteins arranged in these assemblies? *In vivo* studies have shown that mouse SUN1 forms higher-order oligomers in the nuclear envelope [18,40]. Molecular dynamics simulations show that SUN1 homotrimers can interact laterally [41]. This has led to a model of SUN1 higher-order assemblies where multiple SUN1 homotrimers bound to KASH proteins in the ONM form an array in the nuclear envelope (Figure 2c). The oligomerization abilities of the germline-expressed SUN3-5 are less well understood than that of SUN1-2, though recent work suggests that SUN3 can form higher-order oligomers in the nuclear envelope, similar to SUN1 [42].

Recently, another model for higher-order LINC complex assemblies has been proposed: a 6:6 heterohexamer model, where the SUN protein homotrimers face each other in a "head-to-head" interaction (Figure 2d) [32]. The head-to-head model derives from the crystal structure of the human SUN domain of SUN1 or SUN2 bound to human KASH peptides, and is consistent with *in vitro* molecular weight and size determination data of purified SUN proteins and bound KASH peptides in

solution [32]. In previously reported crystal structures of SUN2-KASH complexes, SUN homotrimers were packed in a head to head configuration within the crystals, with the hydrophobic patches present on the outside of their KASH lids of their SUN domains interacting with each other [15,21]. In a solution without a membrane present, it would be energetically favorable for these hydrophobic patches to be buried within the 6:6 interface, suggesting that this structure could represent an experimentally derived artifact. In the context of the nuclear envelope, these hydrophobic SUN domain residues would likely be brought into close proximity with the ONM (Figure 2b) [14]. Furthermore, fluorescence fluctuation spectroscopy (FFS) data and molecular dynamics simulations support a model where in the presence of a membrane, SUN1 homotrimers interact laterally within the nuclear envelope [40,41]. This allows the KASH peptides to easily reach the ONM (Figure 2c, inset). Dual-color FFS data of mouse SUN2 and the KASH peptide and transmembrane domain of mouse nesprin-2 support the model of the nesprin-2 KASH peptide binding to a homotrimer of SUN2 [43]. The head-to-head model also introduces a new issue: for the SUN domains to be angled for their head-to-head interaction, some KASH peptides would not be able to reach the ONM (Figure 2d, inset). Given the current data, we favor a model where SUN and KASH proteins interact in a 3:3 ratio with their interfaces immediately adjacent to the surface of the ONM. The discrepancies between these two models of higher-order LINC complex assembly highlight the need to further investigate the arrangement of LINC complexes in the nuclear envelope.

Regulation of LINC complex disassembly

Intact LINC complexes allow the cell to transmit forces from the cytoskeleton to the nucleus [3]. The regulation of LINC complex disassembly is one mechanism that the cell may use to tune force transmission across the nuclear envelope. For example, when human mesenchymal stem cells are placed under intense cyclic tensile strain, levels of SUN2 protein decrease, thus protecting the chromatin from these forces [44]. Increased mechanical force does not always result in reduced SUN protein levels - for example, increased cytoskeletal stiffness and increased extracellular matrix stiffness can promote SUN protein stability in some cell types [45,46]. The regulation of LINC complex assembly/disassembly in response to various forces seems to depend on specific cellular contexts. In the case of LINC complex disassembly, there are a variety of models for how it is regulated. The AAA + ATPase torsinA is proposed to disassemble LINC complexes, though the exact aspect of LINC assembly it disrupts is unclear (Figure 1b) [47]. Increased calcium levels can also promote the disassembly of SUN homotrimers or promote the SUN-KASH interaction depending on the cellular context (Figue 1b) [17,25,48].

The proteasome has been shown to selectively degrade specific components of the LINC complex, including human SUN2 [49,50,51**−53]. SUN2 is a substrate of the Skip, Cullin, F-box-containing β-transducin repeat-containing (SCF^{βTrCP}) E3 Ligase (Figure 1a) [51**,54]. This degradation is specific to SUN2: the nucleoplasmic domain of SUN2, but not SUN1, contains a non-canonical recognition site for the SCF^{βTrCP} ubiquitin ligase [51**]. Serines found near this site must be phosphorylated for SUN2 to be ubiquitinated [51**]. The phosphorylation of these serines is positively regulated by casein kinase 2 (CK2), and negatively regulated by the phosphatase C-terminal domain NE phosphatase 1 (CTDNEP1) (Figue 1a) [51**].

CTDNEP1 also regulates the proteasomal degradation of SUN2 through its ability to alter the lipid makeup of the INM [52]. When CTDNEP1 levels are decreased, the levels of diacylglycerol in the INM are also decreased, reducing lipid packing in the INM [52**]. This affects SUN2's association with the INM because SUN2 has an amphipathic helix in its nucleoplasmic domain [52**,55]. This helix binds to lipid membranes *in vitro* [52**]. Amphipathic helices tend to bind to membranes with packing defects, so the current model is that SUN2's weakened association with the INM promotes its degradation [52**]. This is also specific to SUN2, as SUN1 does not have this amphipathic helix [52**].

It is interesting that SUN2 is subjected to multiple layers of regulation, but there is little information about human SUN1 being regulated by proteasomal degradation. Given that SUN1 more readily forms higher-order assemblies in the nuclear envelope [18,40,41], one would expect that the cell would have a regulatory mechanism to tune the amount of SUN1 present within the nuclear envelope, and thus regulate when and where these larger assemblies would be able to form.

Proteasomal regulation is also applied to KASH proteins, including the giant isoform of nesprin-2 (nesprin-2G). When the nuclear envelope transiently ruptures during interphase in response to stress caused by cytoskeletal forces, the endosomal sorting complex required for transport (ESCRT) machinery binds to the rupture to re-seal the nuclear membranes [56,57]. BROX, a Bro1 domain protein that binds ESCRT III [58], is brought to the site of nuclear rupture, where it then ubiquitinates nesprin-2G [59**]. The proteasomal degradation of both SUN2 and nesprin-2G may assist in regulating the local forces that act upon the nuclear envelope. Degradation of nesprin-2G near nuclear envelope rupture sites allows for the rupture to be repaired more quickly — it is hypothesized that reducing the amount of mechanical stress at these sites by reducing nesprin-2G levels makes nuclear envelope repair easier [59**]. When the levels of SCF^{βTrCP} E3 ligase are depleted, nuclei

become misshapen [54]. Moreover, the expression of a non-degradable version of SUN2 results in the appearance of wrinkled nuclei [51**].

Degradation of LINC complex components is important for the cell to balance forces across the nuclear envelope. This type of regulation is not limited to human cells — the SUN protein Koi in Drosophila is also ubiquitinated and degraded by the proteasome [60]. Therefore, the specific degradation of LINC complex components is likely a general mechanism used by cells to regulate when and where specific LINC complexes are present within the nuclear envelope.

Conclusion

The LINC complex field is rapidly discovering an increasing array of regulatory mechanisms that cells use to control how LINC complexes are built and taken apart. This is generating many more questions about LINC complex assembly: How are the many different SUN and KASH proteins spatially arranged into functional complexes in the nuclear envelope? Which cellular conditions promote LINC complex assembly? How do cells dismantle or re-arrange LINC complexes? Uncovering new mechanisms, and deepening our understanding of currently studied mechanisms, will allow us to better understand the biology of the LINC complex and the many cellular processes it is involved in.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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* of special interest
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