Perspective

Components of the Linker of the Nucleo- and Cytoskeletal Complex (LINC) as Novel Molecular Targets for Small Cell Lung Cancer

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ABSTRACT

Small cell lung cancer (SCLC) is a recalcitrant cancer and likely due to environmental insults as nearly 90% of the patients have tobacco exposure. Chemotherapy is the standard of care but it rarely affects complete cures and drug resistance is common. While a vast number of clinical trials with both cytotoxic and molecularly targeted therapies have been conducted, no new agents for SCLC patients have reached oncology practices since the 1980s. Thus, there is a pressing need to identify novel molecular targets and effective therapies. In this Perspective, an overview of the putative source cell for SCLC, the neuroepithelial cell, will be presented to aid in the selection of novel drug targets. In particular, neuroepithelial cells are chemosensory and mechanosensory in function, regulating responses to oxygen tension, carbon dioxide, pH, and stretch. A panopoly of secretory factors, both peptidinergic and adrenergic, are released and in numerous experimental systems, shown to communicate with efferent neurons. Because it would be expected that the components of signal transduction pathways active in such functions are intimately connected to cell survival, these proteins are potential drug candidates. One example would be the complex set of proteins residing in the nuclear envelope, the LINCs, (or linkers of the nucleo- and cytoskeletal complex) which provide physical and biochemical signals to and from the extracellular environment and the nucleus. Furthermore, cell-based high content screens are amenable to discovery efforts targeting LINCs.

Keywords: small cell lung cancer, linkers of the nucleo- and cytoskeleton complex, neuroepithelial bodies, nesprins, COSMIC database, molecular targets

Fifteen percent of all lung tumors are classified as small cell lung cancers (SCLC) and approximately 35,000 cases per year occur in the United States and 180,000 cases per year worldwide (1). Characteristically neuroendocrine in nature, a large proportion (two thirds) of patients are diagnosed with extensive disseminated disease with a two-year survival rate of approximately 5% (2). In those patients with limited disease, surgery, combination chemotherapies, and radiotherapies may improve survival. Elderly males with smoking histories are at high risk for the histopathologic diagnosis of these malignant tumors with small cells, limited cytoplasm, and granular, hyperchromatic nuclei. Interestingly, large cell neuroendocrine lung tumors also possess features in common with SCLC and include p53 mutations, disruption of the Rb pathway, and apoptosis resistance. Furthermore, large cell genetic signatures (mRNA expression) are similar to patient biopsies with a strictly small cell phenotype (3,4).

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Combined platinum-based chemotherapy and radiotherapy are first line treatments for all stages of disease but depend on performance status (2,5). Early responses can occur and these are associated with a 25% five-year survival. However, the responses are not durable and drug resistance develops. Second-line therapy has little benefit (2). A significant number of Phase I, II, and III SCLC clinical trials have been undertaken with investigational agents that inhibit the cell cycle, angiogenesis, and multidrug resistance resulting in negative outcomes at worst, or marginal gains in a small number of patients (6,7). Thus, a great need exists to improve outcomes for patients with SCLC as few advances have occurred since the 1980s.

The aim of this Perspective is to offer a hypothesis addressing novel molecular targets for SCLC, those of the linkers of the nucleo- and cytoskeleton. Currently, no such molecular probes or drugs exist for these essential proteins. Expanding the notions of druggable targets may lead to surprising success. As with all overviews of this type, laboratory validation will be needed before drug discovery campaigns are undertaken.

SCLC and Neuroepithelial Bodies (NEB)

The presence of neuroendocrine markers such as chromogranin, synaptophysin, and CD56 (neural cell adhesion molecule 1, NCAM1) aides in the histopathologic diagnosis of SCLC and suggests that the cell of origin resides in the pulmonary neuroepithelial bodies (NEB), a tight cluster of cells residing in the lung epithelia. Genetic signatures (8) also underscore the neuroendocrine nature of SCLC and, in some instances, associate with poor prognosis (3,9). A preliminary analysis found that approximately 60 SCLC tumor lines have gene signatures that overlap with SCLC biopsy samples, and; these, too, have a significant enrichment for genes that are neuroendocrine in nature (unpublished data, Mertins, Lucas, and Ravichandran). But the most convincing evidence derives from p53-/- and Rb-/- genetically modified murine models that develop SCLC when cell-restricted adenoviral vectors effectively transform neuroendocrine pulmonary cells and not Clara cells. Alveolar type 2 cells similarly treated were less efficiently transformed and mortality was limited (10,11). Therefore, NEB remain viable candidates for the cell of SCLC origin, but other sources are possible, including but not limited to pluripotent stem cells (12).

NEB reside in the pulmonary epithelia in interspersed clusters of approximately 20 cells or as single neuroepithelial cells (Figure 1A-B, and for a thorough review, see (13)). In both instances, innervation with afferent and efferent neurons suggested crosstalk between NEB, neurons, and local smooth muscle cells and was demonstrated in animal models (14,15). Functionally, NEB are highly plastic and satisfy many demands of the organism. In the fetal lung, precursors of NEB regulate growth and differentiation, and, in the perinatal lung, adaption to the oxygen-enriched environment. Finally, in the adult, NEB respond to chemical and mechanical stimuli that results in slow and long term adaption, consistent with the non-myelinated status of the connected neurons.

As gas sensors, NEB regulate blood flow and respiratory rate. For example, through oxygen sensing, hydrogen peroxide is generated by a member of the NADPH oxidase family proteins as an intracellular mediator of potassium channel gating. In normoxic conditions, slow acting potassium channels remain open. Under hypoxic conditions, these channels close and further trigger exocytosis and neurotransmitter release. Carbon dioxide levels and acidosis similarly trigger serotonin release in model NEB cultures, the SCLC tumor line, NCI-H146. How detection by these latter stimuli occurs is largely unknown.

Because mechanosensing is essential for normal pulmonary growth and development, these physical stimuli are likely to be critical for NEB cell survival. Interestingly, serotonin release following stretch utilizes distinct transduction pathways from those responding to hypoxia and appears to release cytoplasmic serotonin rather than that in dense vesicles (Figure 1B). Sources of physical stimuli include the underlying airway smooth muscle, fluid flow, and other neighboring cells.

SCLC and Spheroids

SCLC growth characteristics *in vitro* are atypical as most tumor line models are adherent on tissue culture plastic. Early SCLC tumor line establishment revealed that morphology includes floating clusters, aggregates, and well-defined spheres with and without an adherent subpopulation (16). And thus, the propensity to form cell-cell contacts *in vitro* is consistent with the origin of SCLC, the NEB that generally reside in dense regions of 20-25 cells in the pulmonary epithelium.

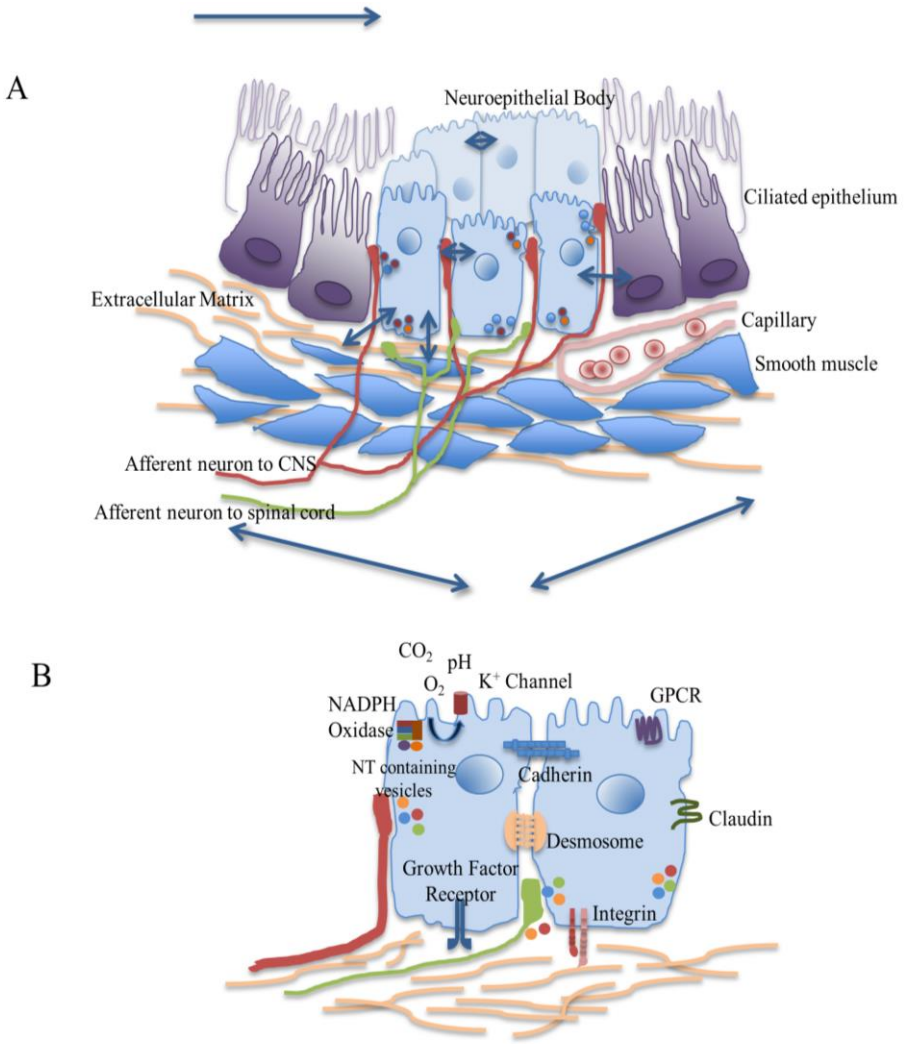

Figure 1. Neuroepithelial Bodies (NEB) in the Pulmonary Epithelium. NEB are localized to the pulmonary epithelium near branch points in the airway. Single cells are also found. The clusters are surrounded by ciliated epithelium and Clara cells (not pictured). Significant innervation is present. Vesicles contain neurotransmitters such as serotonin, regulatory peptides such as bombesin, and neuroendocrine markers. A. Physical forces are omnipresent as depicted by the arrows and include: fluid flow in the airway, cell-cell contact among neighboring NEB cluster cells, cell-cell contact with ciliated epithelium, neurons and smooth muscle, and cell-matrix contact. B. Selected adhesive contacts (cadherin, integrin, claudin, desmosome), receptors (growth factor receptor), and functional proteins in NEB cells. G-protein coupled receptors (GPCR) are present as well as potassium channels (K+ Channel) gated by intracellular hydrogen peroxide levels generated by NADPH oxidase. NEB also release neurotransmitters (NT) such as serotonin upon mechanical stimulation. Figure 1A was adapted from (12) under a license with Elsevier.

Furthermore, adhesion to substrate may depend on environmental factors in one SCLC tumor line (17). As expected, adhesion alters the phenotype as higher clonogenicity (18) can be demonstrated as well as chemoresistance (19). The cell-extracellular matrix receptors, integrins, are upregulated upon adhesion to collagen and fibronectin in the SCLC tumor lines NCI-H82 and NCI-H592 and may promote proliferation and apoptosis resistance (20). Because SCLC *in vitro* morphology parallels the NEB microenvironment, it is likely that adhesion (to both cells and the extracellular matrix (ECM)) and the subsequent effector mechanotransduction pathways regulate survival and thus offer insight into new molecular targets.

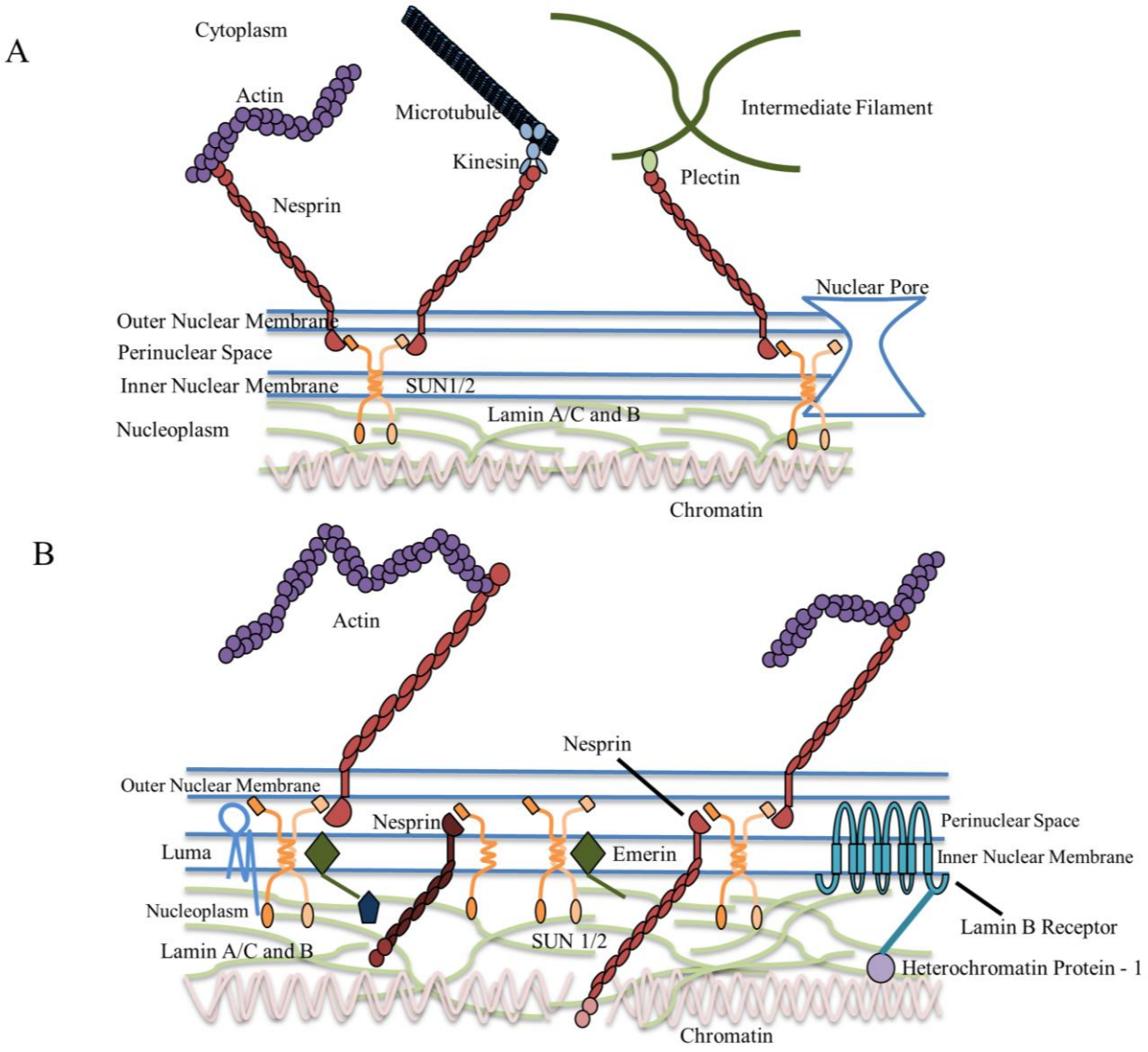

Figure 2. LINC Complex. The LINC complex bridges the cytoskeleton and the nucleoskeleton. It is likely that extracellular biochemical and physical signals first transmitted to the cytoskeleton are then relayed to the nucleus through LINC proteins. In turn, gene expression may be regulated by changes in the nuclear lamina. A. LINC binding to the cytoskeleton. B. LINC proteins as bridges and entities in the inner nuclear membrane that may alter chromatin organization. Figure 2 is adapted from (27) under the Creative Commons License 3.0. Autointegration factor (BAF) is not labeled but depicted (dark blue pentagon) and shown to interact with emerin (EMD) and the nuclear lamina. LUMA is TMEM43.

Mechanosensing and Transcriptional Regulation

It is now clear that the dynamic interplay between tumor cells and the tumor microenvironment is critical for initiation and progression of carcinogenesis. Tumor type is likely to influence the composition of the ECM and thus, alter forces applied to cells *in situ*. Furthermore, tumor cells respond with balancing forces that accommodate proliferation, cell death, infiltrating stromal, and immune and vascular cells (21). Integrins, receptors for ECM components, and cadherins, receptors for cell-cell contact, transduce both biochemical and physical stimuli intracellularly (22).

Classically, integrins cluster at sites of applied forces, activate focal adhesion kinase leading to tension-induced conformational changes, and trigger appropriate downstream signals for cell function. The cytoskeleton plays a critical role as it is reorganized as well.

Several reports in the literature now demonstrate the intimate connection between mechanosensing and transcriptional regulation (23). For example, MRTF-A, a cardiac transcription factor responsive to tensional signals, translocates to the nucleus following the canonical Rho GTPase activation and actin fiber formation. Notably, MRTF-A binds actin monomers and is no longer sequestered cytoplasmically following polymer formation (24). In contrast, transcription factors of the YAP/TAZ family reside in the nucleus to promote cellular responses to a rigid ECM and exit the nucleus when a softer ECM is present (25). While the precise details of these events are not well understood, these examples underscore a role for mechanosensing modulating cellular transcriptional programs.

The nuclear envelope provides a significant barrier to signals emanating from the plasma membrane; however, it must be breached for transcriptional programs to be activated. Currently, it is understood that nuclear architecture, per se, influences gene expression through the establishment of transcriptional factories (26). Generally, they reside interiorly as euchromatin and sites of suppressed gene expression (heterochromatin) remain anchored at the nuclear envelope. The mechanisms of such subcellular movement within the nucleus are not well understood but cellular machinery present in the nuclear envelope may be responsible. Furthermore, these same complexes connect both the cytoskeleton and nucleoskeleton and offer the necessary intracellular bridge.

Linkers of Nucleo- and Cytoskeleton Complex (LINC)

The nuclear envelope (NE) maintains structural integrity, acts as a barrier but with limited permeability through nuclear pores, and modulates transcriptional regulation. Historically, many of its proteinaceous components have been identified through genetic diseases such as Emery-Dreifuss muscular dystrophies (EDMD) (emerin) and Hutchinson-Gilford progeria syndrome (lamin A/C). Critically, extracellular signals, whether biochemical or physical, must be relayed across the NE and the apparatus that does so is described below. While the nuclear pore (NP) remains one site of entry for a myriad of biochemical signals, the LINC complex bridges the cytoskeleton and nucleoskeleton across the perinuclear space (PNS) and is the likely means by which physical forces are sensed (27). Ultimately, the resulting signal transduction (whether intra- or extracellularly) promotes cellular functions such as nuclear positioning during homeostasis and migration, localization of the centrosome during cell division, and chromosome tethering to the spindle body.

The NE is a bilayered membrane, contiguous with the endoplasmic reticulum, and dotted with NPs (Figure 2). The inner nuclear membrane (INM) is supported by the nuclear lamina residing in the nucleoplasm through multiple transmembrane proteins such as the lamin B receptor (LBR). These, in turn, may also connect to the nuclear lamina and chromatin indirectly via proteins such as heterochromatin protein-1 (HP-1). The outer nuclear membrane (ONM) interfaces with the cytoplasm and contains transmembrane proteins that contact both the cytoskeleton and span the PNS that further contact proteins residing in the INM. The cytoskeletal contacts are varied as the ONM transmembrane proteins can bind actin, microtubules, and intermediate filaments with isoforms (large and small) of the Nesprin family.

Structural Components of the LINC Complex. There are numerous protein families that comprise the LINC complex and, within each family, multiple isoforms exist. They are summarized below and depicted in Figure 2A-B and Figure 3. The reader is directed to recent reviews that provide an in depth discussion of structure and function (28,29). It is notable that the protein families are well-conserved as homologous proteins exist between *C. elegans* and *D. melanogaster* and mammals (rodents and humans). Tissue distribution is varied; some are universally present (e.g. emerin), while others are uniquely expressed (e.g. KASH5 in the testes).

Nesprins. The generalized nesprin contains an N-terminal actin binding domain (or calponin homology domain, CH), multiple spectrin repeats, followed by a transmembrane helix and a domain that binds to SUN proteins, KASH. This description is apt for Nesprins that reside in the ONM. In contrast, INM nesprins may bind to the nuclear lamina proteins, lamin

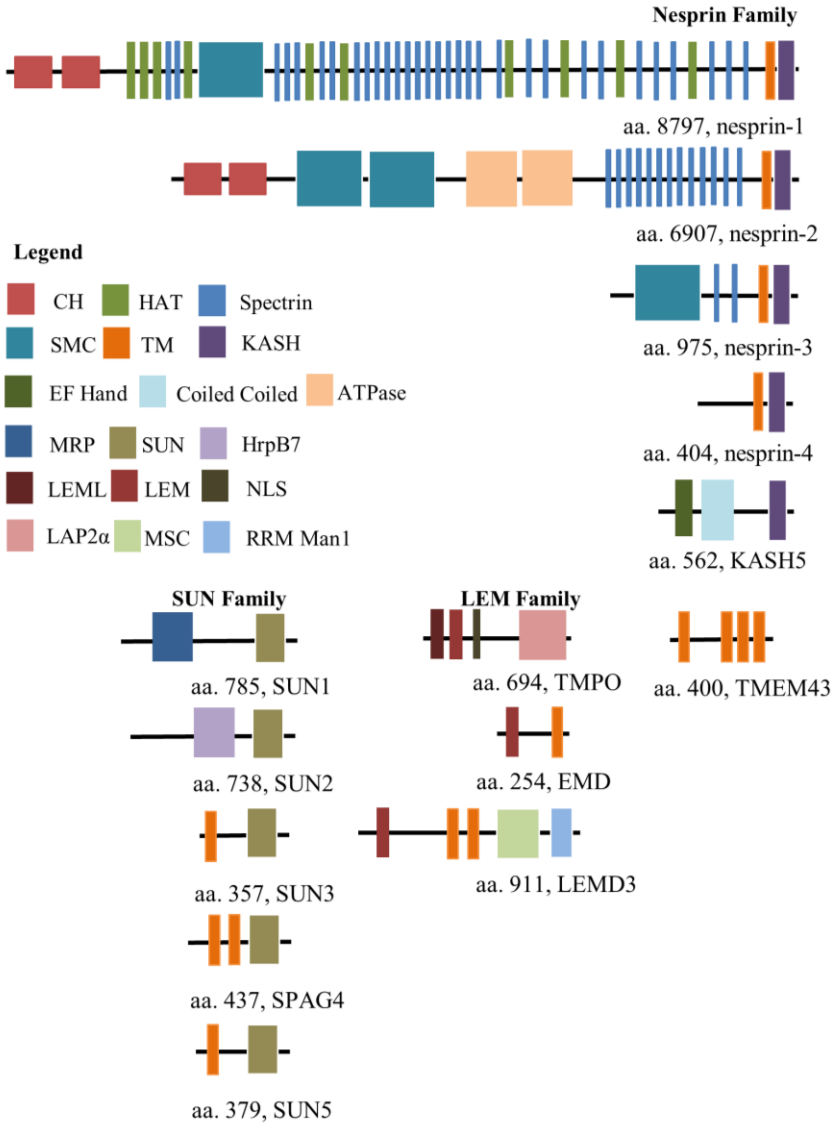

Figure 3. Conserved Protein Domains of LINC Complex Members. The Gene and Protein database found at the National Center for Biotechnology Information was searched for LINC complex members (http://www.ncbi.nlm.nih.gov) described in the text. Protein domains defined by the Conserved Domain database are depicted. While the length of each protein in amino acids is indicated, scale is approximate. The gene symbols for LAP2α, emerin, and MAN1 are TMPO, EMD, and LEMD3, respectively. CH – calponin homology domain/actin binding domain, HAT – Half-A-TPR repeat, SMC – chromosome segregation domain, TM – transmembrane domain, KASH – Klarsicht, ANC-1, and Syne homology domain, EF Hand – EF Hand domain, Coiled Coiled – Coiled coiled region, ATPase – ATPase involved in DNA repair, MRP – Mitochondrial RNA binding protein, SUN – Sad1-UNC domain, HrpB7 – Bacterial type III secretion protein, LEML – LEM like domain, LEM – Lap2/emerin/Man1 domain, NLS – Nuclear localization signal, LAP2α - Lamina associated polypeptide 2α domain, MSC – Man1-Src1p-C-terminal domain, RRM Man1 – RNA recognition motif in inner nuclear membrane protein Man1.

A/C and lamin B, through intermediary proteins as well. In this instance, the spectrin repeats are directed toward the nucleoplasm. Studies in cardiomyocytes with KASH domain-deleted nesprin demonstrate that nuclear architecture is disrupted and heterochromatin is reduced (30). It is of interest to note that mice missing Nesprins-1 and -2 die at birth due to respiratory failure (31).

SUN proteins. This family of INM proteins, named for its founding members, Sad in *S. pombe* and UNC-84 in *C. elegans*, are fairly versatile in their interactions with other LINC members. Structurally, the N-terminus contacts the nuclear lamina, followed by coiled-coiled domains and a KASH-interacting domain, the SUN domain. The penultimate and final domains extend into the PNS. SUN1 and SUN2 may exist as homo- or heterodimers or trimers (32). Confirmation of SUN protein binding to nesprins was obtained in crystal structures that demonstrate extensive hydrogen bonding and SH bridges (33). SUN proteins (of which at least 5 isoforms are known) aid in the localization of ONM nesprins (34) and are relocated to the Golgi apparatus under conditions in which lamin A/C is absent, a cell-death inducing event for mouse embryo fibroblasts (MEFs) (35). In lamin A/C/SUN1 double knockout mice, perinatal lethality was associated with deflated lungs (36).

LEM-domain containing proteins. LEM domains are named for the INM proteins LAP2, emerin, and MAN1 and interact with chromatin through attachment to BAF (barrier-to-autointegration factor). The gene symbols for LAP2α, emerin, and MAN1 are TMPO, EMD, and LEMD3, respectively. Notably, this family of proteins binds transcription factors such as SMADs and E2F, suggesting a function beyond nuclear structural integrity (37). Emerin has been extensively studied owing to its mutational status in patients with EDMD (38). Ubiquitously expressed, this integral INM protein extends its N-terminus into the nucleoplasm, where contacts are made with the lamina and chromatin. A transmembrane domain follows and terminates in a short series of residues that reside in the PNS. Interaction partners of emerin in the INM include nesprins, SUN proteins, and LUMA (see below). Numerous studies on emerin structure and function have delineated the consequences of the mutational events and are not limited to skeletal or cardiac muscular tissue. For instance, emerin deficient MEFs have disrupted nuclear morphology and impaired responses to mechanical stress (39,40). Under strain, apoptosis occurs. Attempts to recapitulate EDMD in mice have not been fully successful, suggesting the expressed phenotype results from a multigenic condition (41).

TMEM43 (transmembrane protein 43). TMEM43 (also known as LUMA) is a highly conserved INM integral membrane protein with orthologs in both prokaryotic and eukaryotic species (42). It possesses 4 transmembrane domains interrupted by a hydrophilic loop that resides in the PNS. Both the N- and C-termini contavet the nucleoplasm and has been shown to oligomerize via the first TM domain. LUMA binds to lamin A/C, lamin B2, and emerin, but not other LEM domain proteins. Functional studies on LUMA are lacking at this time, but its common presence across organisms suggests an essential function. Mutations in LUMA have been found in a family with cardiac disease resulting in sudden death (43).

Nuclear Lamins. While lamin A/C and lamin B, intermediate filament proteins that underlie the INM, are not considered LINCs, their connections to LINCs are multiple and might be considered effectors of mechanical stimuli that cross the NE and originate in the plasma membrane. The role of nuclear lamins cannot be overstated as they provide physical support, modulate chromatin organization, and gene expression (see recent review (44)). A series of reports underscore the role of lamins, associated LINC proteins, and regulation of the cell cycle. In particular, it has been shown that the tumor suppressor RB1 (retinoblastoma 1) is sequestered in the laminar nuclear scaffold along with its binding partner, the transcriptional activator, E2F by LAP2α (lamin associated protein 2α or thymopoetin, (TMPO)). During the transition from G0 to G1 phase, dephosphorylated RB1 releases E2F

Table 1. Cellular Functions of LINCS

LINC Family	Cellular Function	References
Nesprin and SUN	Nuclear Architecture	27, 36-38, 51-53
Nesprin and SUN	Mitosis	54, 55
SUN	Genomic Stability	56, 57
Nesprin and SUN	Centrosome Positioning	58, 59
Nesprin and SUN	Migration	60
Emerin	Mechanotransduction	39, 40

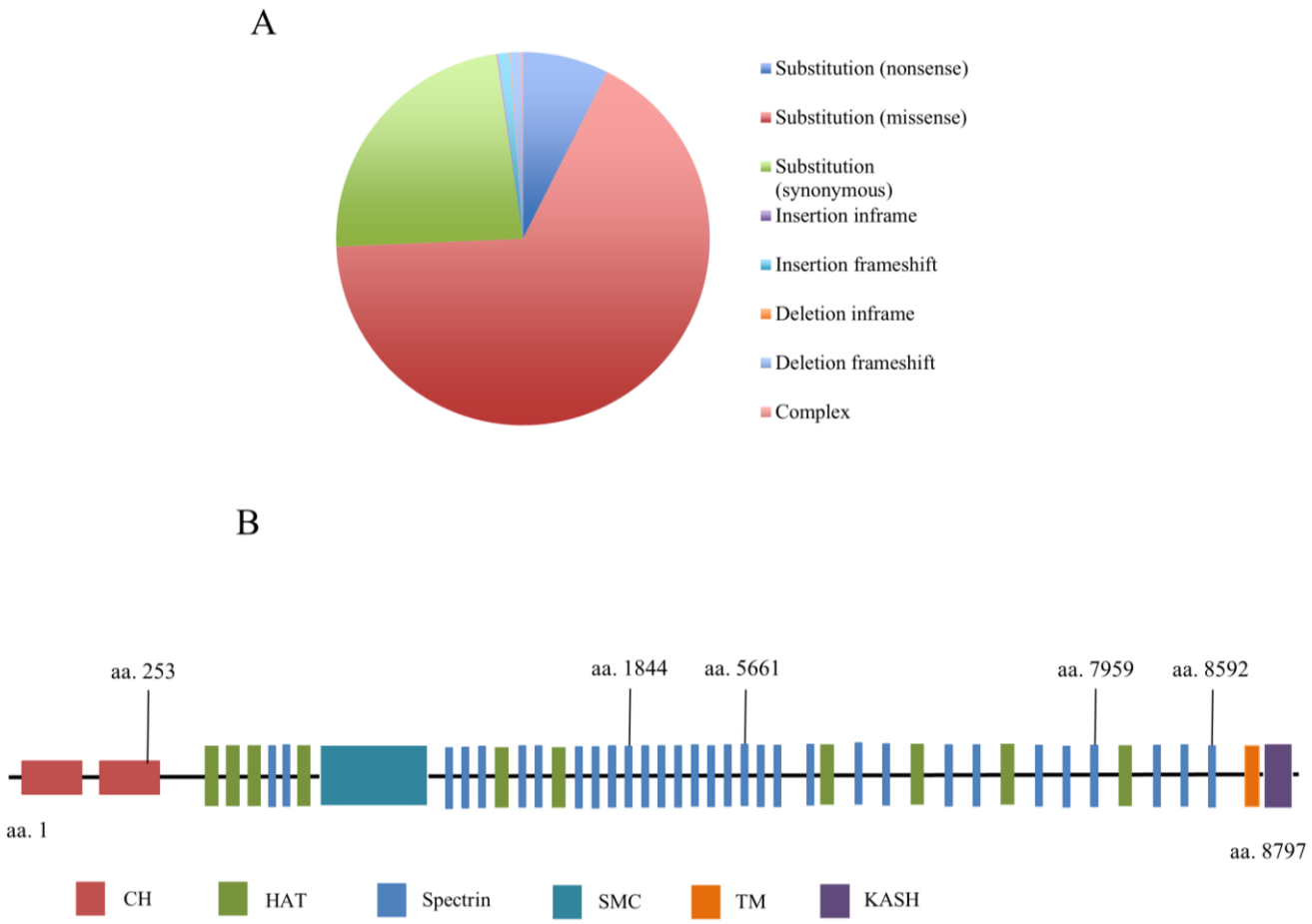

Figure 4. Catalog of Mutations in a Representative LINC, SYNE1 (nesprin-1). A. The majority of mutations in lung tumor clinical samples (all pulmonary histologies including NSCLC, SCLC, and squamous carcinoma) are missense. B. Proteins domains of SYNE1 with confirmed somatic mutations indicated. CH – calponin homology domain (containing an actin binding domain), HAT – Half-A-TPR repeat, SMC – chromosome segregation domain, TM – transmembrane domain, KASH – Klarsicht, ANC-1, and Syne homology domain.

which then allows transcription of cell cycle genes. Of note, RB1 is commonly mutated in SCLC, with one report demonstrating mutations, loss of heterozygosity, and/or complete deletion in 29 of 29 fully sequenced genomes of clinical samples (45). LAP2α protects the inactive RB1-E2F complex from degradation and if functioning, promotes cell cycle arrest (46,47). Knock down of lamin A similarly promotes cell cycle arrest because the RB1-E2F complex is released to the nucleoplasm and becomes available to the degradation apparatus (48). Thus, it remains a formal possibility that the many interactions between LINCs and the nuclear lamina may regulate transcription given their proximity and binding partners. Furthermore, depending on the RB1 mutation, small molecules selective for the RB1/E2F/LAP2α interactions may promote degradation in a manner similar to that described above.

Cellular Functions of LINCs. The presence of structural domains (e.g. coiled-coiled), localization, and binding partners of the LINC complex (actin, microtubules, and intermediate filaments), suggests that, at a minimum, the LINC complex regulates nuclear architecture through structural support. Importantly, numerous other functions have been identified (Table 1). As discussed below, a role for the LINC complex proteins has been demonstrated in mitosis, cytokinesis, centrosome homeostasis, and

mechanotransduction. In the past, a diagnostic and prognostic view of the nuclear lamina in cancer was understood, but it was not well-defined (49). A recent review of the role of the NE, INM transmembrane proteins, and cancer provides background on this topic (50).

Role in Nuclear Architecture. Disrupted SUN/KASH domain interaction resulted in widening of the PNS (51). In addition, emerin-/- fibroblasts had impaired nuclear morphology and response to deformability (39). This finding may suggest that LINC complexes play a role in the crush artifact frequently noted in SCLC biopsy specimens (52). Finally, SUN proteins may organize the nuclear pore complex (53).

Role in Mitosis. In the model organism *C. elegans*, when SUN/KASH domain interactions were prevented, chromosome pairing during prophase was altered (54). This is consistent with the finding that entry into mitosis was delayed. In mammalian cells, upon deletion of SYNE-1, membrane movement during cytokinesis was impaired (55). This finding emphasized the connections between nesprins, kinesins and microtubules.

Role in Genomic Stability. It is notable that loss of lamin A and SUN proteins leads to telomeric shortening and increased genomic instability in mammalian cells (56,57).

Role in Centrosome Positioning. Tethering (58) and migration (59) of the centrosome to locations peripheral to the NE and ONM occurs through microtubules and SUN/Nesprin proteins .

Role in Migration. Intracellular forces that regulate cellular movement and posterior positioning of the nucleus are dependent on SUN proteins and nesprins in polarized and migrating fibroblasts (60).

Role in Mechanotransduction. Relevant to the topic of this review, MEFs deficient in emerin have limited nuclear elasticity and do not respond to mechanostimulation by upregulating genes known to be sensitive to such stimuli (egr-1 and iex-1). Furthermore, these same MEFs are more apoptotic under significant stress stimuli (39,40).

SCLC and the LINC protein, SYNE1 (nesprin-1)

Because LINC complex proteins function in mitosis, cytokinesis, and cell homeostasis and may be essential mediators of physical forces that detect survival signals from cell-cell and cell-ECM contacts, they may be suitable drug candidates for SCLC. Furthermore, NEB, the putative origin of SCLC tumors, normally respond to stretch in the pulmonary epithelium and may signal survival in this fashion. Presently, there is no experimental evidence to suggest that any of LINC proteins are oncogenes.

Table 2. Comparison of Point Mutation Frequency[a] in Selected LINC Complex Members in SCLC and NSCLC[b] Tumor Samples[c]

Gene	SCLC (%) (mutated samples/total samples)	NSCLC (%) (mutated samples/total samples)	p Value[d,e]
SYNE1	29.5 (18/61)	11.3 (70/621)	$<10^{-6}$
SYNE2	18.6 (8/43)	9.2 (74/801)	$<10^{-6}$
SUN1	0 (0/40)	2.3 (19/801)	$<10^{-6}$
SUN2	0 (0/40)	0.9 (7/794)	0.13
EMD	0 (0/41)	0.6 (5/801)	0.004
TMEM43	2.4 (1/41)	0.5 (5/796)	0.0004
TMPO	0 (0/41)	0.5 (4/801)	0.64

[a]Point mutations are categorized as confirmed somatic, previously reported, or variant of unknown source.
[b]NSCLC tumor samples include adenocarcinoma and squamous cell carcinoma.
[c]Data extracted from COSMIC Database on 061415.
[d]χ squared test for independent samples
[e]Expected mutational frequency was calculated from COSMIC Database across all tumor histologies. Mutational frequency varied for each gene and ranged from 0.2% (EMD) to 7.4% (SYNE1)

Table 3. Confirmed Somatic Mutations (COSMIC Database) in SCLC and NSCLC

Tumor Type	Amino Acid	Mutation	Domain
SCLC	P253R	Missense	CH*
	G1844R	Missense	Spectrin
	G5661-	Nonsense	Spectrin
	A7959T	Missense	Spectrin
	L8592F	Missense	Spectrin
NSCLC	R6277L	Missense	Spectrin
	V6472L	Missense	N/A
	I7815K	Missense	N/A

* CH; Calponin Homology

Since the presence of mutations in any of the LINC complex components may suggest a certain survival advantage during cancer progression, the COSMIC database was examined (http://www.sanger.ac.uk/cosmic). The COSMIC (Catalog of Somatic Mutations in Cancer) database is a compendium of mutations (substitutions, insertions, deletions, and copy number variation) found in clinical specimens and cultured tumor lines across the entire histologic spectrum. SYNE1 was selected at random as a representative LINC protein, i.e., without any prior knowledge or analysis of the COSMIC database. The tumor tissue with the highest percent of point mutations was the colon (27%), followed by stomach (26%), esophagus (18%), skin (18%), and lung (14%). It was of interest to examine the 1159 lung tumor samples with mutations. The overall distribution of mutations varied but the large majority (67%) were missense substitutions, 23% were synonymous substitutions, and 7% were nonsense substitutions (Figure 4A). Copy number loss was present in pulmonary cancers, but as a rare event (<1%).

Under- or overexpression of SYNE1 may also portend a role in disease pathogenesis. Therefore, gene expression of SYNE1 in lung tumor clinical specimens was examined in the COSMIC database as well. The question was examined by comparing SYNE1 gene expression levels in both SCLC and non-small cell lung cancer (adenoma) samples. In COSMIC, no differences were found. Furthermore, this was confirmed in the Oncomine Gene Browser (http://www.oncomine.org), a curated database of published gene signatures in clinical samples and tumor lines.

It was of interest to analyze the SYNE1 mutations in SCLC and nonsmall cell lung cancer (NSCLC) in the COSMIC database. For SCLC, 61 clinical samples were tested, of which 18 (29.5%) had mutations while only 11.3% of NSCLC samples were found mutated (70 of 621) (Table 2, $p < 10^{-6}$). An in depth evaluation of the mutations was available and 5 were confirmed somatic for SCLC and 3 for NSCLC (Table 3). Because localization of the mutations to known protein domains may suggest critical survival function to the cancer cell, the mutations were mapped to the SYNE1 protein sequence (Figure 4B). All mutations in SCLC were located in protein domains. Only 1 of the 3 NSCLC mutations was located in a protein domain. Thus, there is a propensity to find SYNE1 mutations in SCLC, suggesting a role for at least one member of the LINC complex in pathogenesis and its potential as a novel molecular target.

A comparison of the point mutation frequency of clinical samples in the COSMIC database was also conducted for other selected LINC complex members (Table 2). Both SYNE2 and TMEM43 were mutated more frequently in SCLC than NSCLC tumors ($p < 10^{-6}$ and $p < 0.004$, respectively). For SUN1 and EMD, the reverse was found. There was no difference in the SUN2 and TMPO mutation frequency between the two tumor types. Thus, based on this limited analysis, some LINC complex members may be better choices as molecular targets. It should be noted that the analysis such as the one presented in Table 2 assumes mutational frequency is reflective of biologic function. This indeed may not be the case and a significant research undertaking would be required to confirm the

functional (survival) roles of SYNE1, SYNE2, and TMEM43 in SCLC.

Proposed Cell-based Screening for Small Molecule Modulators of LINC Complex Proteins

Typically, target validation would precede drug discovery campaigns, whether in research institutions or pharmaceutical laboratories. This indeed would be necessary in the case of LINC complexes. *In vitro* studies demonstrating tumor growth inhibition in knock-down studies would be an essential first step. Xenograft studies of those same modified tumor lines may provide further evidence. However, because the proposed targets are not traditional drug targets that typically have enzymatic activity that can be measured in functional assays, standard drug screens are not possible

In order to identify small molecule inhibitors and/or activators (and unique molecular probes), high content screens with multiparametric output (or cell-based screens) would offer one means to do so (61). In a high-content screen, tumor cells containing GFP-labeled proteins of interest in LINC complexes could be examined for morphologic changes that precede cell death. Examples of such changes may include altered nuclear morphology, mislocalized centrosomes, disrupted spindle apparatus, or widened or reduced PNS. This later change may require counterstaining and careful selection of the targeted protein (62). An alternative approach could examine the loss of spheroid formation in SCLC tumor lines with such growth characteristics along with the above-mentioned alterations as a preliminary effort to link mechanotransduction and the LINC complex. Finally, a recent report demonstrates feasibility of combining multiparametric high content screening with morphologic output and cytotoxicity measures and this, too, could be utilized for the proposed drug targets (63).

Many, if not, all high content screens are static in nature. In particular, they typically require exposure to the chemical library for a predetermined amount of time and then a biochemical assay and image processing. Any effects of time are determined in secondary screens. In contrast, a dynamic high content screen might be attempted. Because biological processes are kinetic in nature and require significant crosstalk between signal transduction pathways and feedback/feedforward mechanisms, evaluating the effect on small molecules in real time and even at single molecule detail may offer better therapeutics. While there are no such reported screens, with development time and effort, it may be feasible to conduct one as basic biologic assays are available. For example, EGF receptor dimerization upon stimulation has been visualized and kinetic parameters determined (64). Similarly, inhibition of oligomerization of nesprins upon stimulation might be of interest. Because it would be expected that feedback and feedforward loops may generate drug resistance, a lead compound that does not evoke these secondary pathways may be a better therapeutic than one that does. Real-time kinetic evaluations are better suited for such discovery as the may uncover parameters that trigger the secondary crosstalk. Thus, evaluating dynamics in a real-time high content screen offers a new avenue for drug discovery.

Conclusions

SCLC is a recalcitrant cancer that acquires a drug resistance phenotype, is metastatic, and highly invasive within the lung tissue itself, thus, limiting surgical interventions. Uniquely among *in vitro* tumor models, SCLC tumor lines typically form floating cell aggregates, clusters, and spheroids with and without an adherent subpopulation. This suggests the critical nature of cell-cell contact and cell-matrix signals that require significant force balance within such three dimensional structures. Similarly, the putative cells of tumor origin, the NEBs, reside in functional innervated clusters responding to physical stimuli with secretion. Therefore, small molecule therapy that targets the Achille's heel of such signal transduction pathways (i.e., survival signals) might offer effective and rapid therapy that limits drug resistance. Thus, it is proposed that novel molecular targets in SCLC exist in the LINC complex.

As described above, the LINC complex operates in many essential cellular functions, which further emphasizes their selection as potential effective drug targets. For example, LINCs play roles in centrosome positioning and localization, chromosome pairing during mitosis, and cell migration. The latter might be exceptionally critical for the highly invasive SCLC. Lastly, it should be mentioned that nuclear crush artifacts in SCLC histology suggests fragile nuclear architecture, and targeting the LINC complex

under these conditions may bring about the desired outcome, tumor shrinkage.

At present, answers to questions of selectivity and specificity of targeting components of the LINC complex are unknown, but it should be mentioned, that at least for several tumor types, targeting ubiquitous microtubules can lead to cures. But more importantly, preventing normal cellular function in cancer cells may be fundamentally different as they are highly stressed cells and small doses of inhibitors/activators may suffice to trigger cell death. This may not occur in normal cells, where toxic stress is readily contained.

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Abbreviations

BAF, barrier to autointegration factor;
CH, calponin homology;
COSMIC, catalog of somatic mutations in cancer;
ECM, extracellular matrix;
EDMD, Emery-Dreifuss muscular dystrophy;
EMD, emerin;
EGF, epidermal growth factor;
GPCR, G-protein coupled receptor;
HAT, Half-A-TPR repeat;
HP-1, heterochromatin protein-1;
HrpB7, Bacterial type III secretion protein;
INM, inner nuclear membrane;
KASH, Klarsicht, ANC-1, and Syne homology;
LAP2α, lamin associated protein 2α;
LBR, lamin B receptor;
LEM, LAP2, emerin, and MAN1 protein family;
LEML, LEM-like domain;
LINC, linker of the nucleo- and cytoskeletal complex;
LUMA, see TMEM43;
MEF, mouse embryo fibroblast;
MRP, Mitochondrial RNA binding protein;
MRTF-A, myocardin-related transcription factor-A;
MSC, Man1-Src1p-C-terminal domain;
NADPH, nicotinamide adenine dinucleotide phosphate;
NCAM1, neural cell adhesion molecule 1;
NE, nuclear envelope;
NEB, neuroepithelial bodies;
NLS, nuclear localization signal;
NP, nuclear pore;
NSCLC, non-small lung cancer;
NT, neurotransmitters;
ONM, outer nuclear membrane;
PNS, perinuclear space;
RB1, retinoblastoma 1;
RRM Man1, RNA recognition motif in inner nuclear membrane protein Man1;
SCLC, small cell lung cancer;
SMA, chromosome segregation domain;
SMAD, protein family with gene products of *D. melanogaster* gene mothers against decapentaplegic (Mad) and the *C. elegans* gene Sma;
SUN, Sad in *S. pombe* and UNC-84 in *C. elegans*;
SYNE1, nesprin-1;
SYNE2, nesprin-2;
TAZ, tafazzin;
TM, transmembrane domain;
TMEM43, transmembrane protein 43 or LUMA;
TMPO, thymopoetin;
YAP, yes-associated protein

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