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Tissue specificity in the nuclear envelope supports its functional complexity

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Keywords: NET, tissue specific, laminopathy, nuclear envelopathy, nuclear envelope, NPC, cell cycle regulation, spatial genome organization, cytoskeleton

Nuclear envelope links to inherited disease gave the conundrum of how mutations in near-ubiquitous proteins can yield many distinct pathologies, each focused in different tissues. One conundrum-resolving hypothesis is that tissue-specific partner proteins mediate these pathologies. Such partner proteins may have now been identified with recent proteome studies determining nuclear envelope composition in different tissues. These studies revealed that the majority of the total nuclear envelope proteins are tissue restricted in their expression. Moreover, functions have been found for a number these tissue-restricted nuclear envelope proteins that fit with mechanisms proposed to explain how the nuclear envelope could mediate disease, including defects in mechanical stability, cell cycle regulation, signaling, genome organization, gene expression, nucleocytoplasmic transport, and differentiation. The wide range of functions to which these proteins contribute is consistent with not only their involvement in tissue-specific nuclear envelope disease pathologies, but also tissue evolution.

Introduction

The nuclear envelope (NE) was historically viewed as little more than a physical barrier, like the walls of a mediaval city (Fig. 1). Such cities typically had a double wall or moat for protection and accordingly the NE is comprised of two separate lipid bilayers, the inner and outer nuclear membranes (INM and ONM) with a lumen in between.1 That the NE would have two “walls” vs. the single lipid bilayer of the plasma membrane reinforced the basic functional view scientists had of this structure to protect the all-important genome. It has been proposed variously that the single lipid bilayer of the plasma membrane reinforced the functional complexity for the NE began to be realized with the discovery that both the INM and ONM contain a variety of NE transmembrane proteins (NETs). The first NETs were identified starting in 1988 by microscopy screening, genetic and biochemical means, mostly based on associations with the lamin polymer. Novel NETs continued to be discovered at a pace of one per year (reviewed in ref. 11) until their number grew exponentially with the application of proteomic approaches.

Nuclear envelope links to inherited disease gave the conundrum of how mutations in near-ubiquitous proteins can yield many distinct pathologies, each focused in different tissues. One conundrum-resolving hypothesis is that tissue-specific partner proteins mediate these pathologies. Such partner proteins may have now been identified with recent proteome studies determining nuclear envelope composition in different tissues. These studies revealed that the majority of the total nuclear envelope proteins are tissue restricted in their expression. Moreover, functions have been found for a number these tissue-restricted nuclear envelope proteins that fit with mechanisms proposed to explain how the nuclear envelope could mediate disease, including defects in mechanical stability, cell cycle regulation, signaling, genome organization, gene expression, nucleocytoplasmic transport, and differentiation. The wide range of functions to which these proteins contribute is consistent with not only their involvement in tissue-specific nuclear envelope disease pathologies, but also tissue evolution.

form 10 nm wide filaments from the stacking of coiled-coil dimers in linear arrays,2 more like the fibers of a rope than the building block structure of actin filaments and microtubules. Though differing thus from rigid city walls this structure is actually stronger because actin filaments and microtubules will rupture under stresses that leave intermediate filaments, which can stretch to three times their length, intact,3 and so this structure may be of greater benefit to the nucleus as we now know it—a dynamic organelle under considerable mechanical pressures from chromatin on the inside and cytoskeletal connections on the outside.4 Lamins were likely the original intermediate filaments and are highly conserved in evolution among higher eukaryotes, but not so much as this stabilizing function: recent reports indicate that nuclearized organisms previously thought to lack lamins such as Dictyostelium and Trypanosoma brucei have functional homology with this coiled-coil based structure.5-8 The gates of the city are the nuclear pore complexes (NPCs), large macromolecular assemblies that form transport channels at places where the ONM bends in to fuse with the INM. NPCs are built from 30 core components, called nucleoporens or Nups that are present in multiple copies according to the 8-fold symmetry of the assembled structure.9,10 All the above appeared to be still consistent with the idea of the NE as just a protective barrier, but in a mediaval city some of the most important activities, from the coordination of roads to the sentries to the markets and general commerce, took place at the walls or just inside the gates. Accordingly, a greater functional complexity for the NE began to be realized with the discovery that both the INM and ONM contain a variety of NE transmembrane proteins (NETs). The first NETs were identified starting in 1988 by microscopy screening, genetic and biochemical means, mostly based on associations with the lamin polymer. Novel NETs continued to be discovered at a pace of about one per year (reviewed in ref. 11) until their number grew exponentially with the application of proteomic approaches a little over a decade later.11,12 The study of NETs and lamins in the past 20 years has now linked the NE to functions ranging from cell and nuclear mechanical stability to cell cycle regulation and stem cell maintenance, signaling cascades, genome organization and gene expression.
Lamins and several of the earlier discovered NETs have also been linked to many human diseases. These range from muscular dystrophies, to lipodystrophies, cardiac and neuropathy, dermopathy, osteoarthropathies (isolated, together with melorheostosis or as a symptom of Buschke-Ollendorff syndrome), dystonia, and premature aging syndromes. A potential resolution can be found in the “guilt by association” hypothesis that disease-causing mutations in near ubiquitous proteins in the NE cause diseases restricted to specific tissues? This idea is supported by observations that many disease-linked NE proteins appear to function in complexes and that few have specific enzymatic functions themselves that could result in pathologies. Apart from the structural functions of the lamin and NETs of the SUN and nesprin families, the proteins thus far mutated in NE diseases have few inherent functions. Only LBR, which is mutated in the bone disorder Greenberg skeletal dysplasia, has been shown to have an enzymatic activity—that of a sterol C-14 reductase. Other NETs linked to disease have no known enzymatic functions, but instead appear to influence a wide variety of activities through their binding partners, of which they have a great many. Indeed, observations that Emery-Dreifuss muscular dystrophy (EDMD) can be caused not only by lamin A mutations, but also by mutations in its interacting partners emerin and plectin, demonstrate that these proteins are part of larger complexes that yield disease when disrupted and support the “guilt by association” hypothesis. This idea is also supported by observations that NE-linked diseases tend to be genetically heterogeneous, with at least 19 variants described thus far for limb-girdle muscular dystrophy and for Charcot-Marie-Tooth disease. If unidentified tissue-specific components of large NE protein complexes do indeed mediate the tissue-restricted disease pathologies they could themselves potentially cause additional disease variants.

### Nuclear Envelope Proteome Tissue Specificity

As a first step to attempt to identify candidate proteins that mediate tissue-restricted NE disease pathologies, new proteomic studies were undertaken on NEs isolated from different tissues. The first study determined the NE proteome of a lymphocyte-enriched peripheral blood leukocyte fraction. Fluorescence activated cell sorting (FACS) revealed that the fraction was roughly 75% T-cells and helper T-cells. The cells from each blood donor were divided in two fractions. One was activated by treatment with phytohemagglutinin, because an enormous amount of dense peripheral chromatin can be observed by electron microscopy at the NE in the untreated cells and this largely dissipates upon such activation. Thus it was expected that some differences in protein composition at the periphery must occur to direct the visual differences in attached chromatin. Indeed, not only were there many proteins identified that had not been found in the original proteomic studies and which were preferentially expressed in blood or blood specific according to transcriptome data, but differences were observed in NET composition even between the two states of the same cells from the same donors (Fig. 2A). Moreover, in keeping with the idea that some of these differing proteins would contribute to chromatin organization, visual screens identified different sets of blood-specific NETs that could promote chromatin compaction or after spatial genome organization.

A second study used identical conditions for extractions and mass spectrometry to investigate the NE proteome of skeletal muscle. Whereas the lymphocyte nuclei are round and have a very large amount of dense peripheral chromatin, most of the muscle nuclei are oval, relatively flattened, and have an intermediate amount of dense peripheral chromatin. Moreover, there is a greater tendency for centromeres to be at the nuclear periphery in differentiated muscle cell nuclei, further suggesting that there would be differences in NE INM protein composition. There are likely to be differences in the ONM as well because the lymphocytes have a single nucleus and a relatively small cytoplasm whereas muscle cells are syncytial with many nuclei in an individual cell. The muscle NE proteome also included many proteins that were not found in other NE proteomic studies and, again, transcriptome analysis indicated that many of these proteins are either preferentially expressed in muscle or muscle-specific.

The liver NE proteome had been analyzed previously, but could not directly be compared with the leukocyte and muscle studies because these later studies had many more biological and technical replicates using more sensitive mass spectrometers as well as using multiple sequential proteolytic digests, which was found to increase identification of transmembrane proteins presumably lost otherwise because of aggregation of hydrophobic regions. Thus, a third study used these same conditions to re-examine the proteome of liver NEs. The new liver NE proteome identified roughly 2½ times more proteins compared with the original study; however, these proteins for the most part were not ones found in the leukocyte and muscle studies. Instead, many were like those found in the original study, preferentially expressed in liver according to the transcriptome data.

The preferential tissue expression of many of the predicted transmembrane proteins identified in the three studies was directly confirmed by both RT-PCR and western blot where antibodies were available. Moreover, staining of rat tissue cryosections with these antibodies demonstrated that those found by proteomics only in a particular tissue yielded the characteristic “rim” staining of the NE only in that tissue, while only background staining was observed for the tissues where it was not found by the mass spectrometry (Fig. 2B). This confirmed both their tissue-specificity and their qualification as NETs. All in all, less than 20% of the total NE proteins identified and a similar number of the putative NETs identified were found in the NEs of all three tissues (Fig. 2C). This is especially remarkable given that none of the three tissues investigated represented a homogeneous population of single
cell types. Blood leukocyte-enriched populations were >75% lymphocytes, which were mostly T-cells and helper T-cells but also included cytotoxic T-cells and B-cells, yet the populations also included 5–20% myeloid cells. Muscle contains connective tissue, nerves and blood vessels, not just myocytes. Similarly, liver may be comprised of about 80% hepatocytes, but it also contains nerves and blood vessels, blood cells and fat cells. Thus, it might have been expected that the cell types shared between different tissues would bias the results toward overestimating the proportion of shared proteins among the sampled tissues. Many of the newly identified NETs appear to be very specific to the cell types and tissues in which they were found while others are expressed in a subset of tissues according to transcriptome data; for simplicity we will use the term “tissue specific” henceforth.

The unexpectedly high degree of tissue-specificity observed raised the concern that contaminants from other organelles might be contributing to the tissue differences. Such potential contaminants can be estimated based on proteome studies of other organelles and GO-functional/ subcellular localization annotations from the Gene Ontology database. The major membrane contaminants of NE preparations would be expected

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**Figure 1.** The medieval nuclear envelope. Historically the nuclear envelope (NE) was viewed as little more than a barrier, like the walls of a medieval city. The NE has a double membrane structure with inner (INM) and outer (ONM) membranes and the ONM is continuous with the ER. An intermediate filament lamina polymer underlies the INM, giving it stability, and is connected to the INM by several NE transmembrane proteins (NETs).
subcellular localizations. This idea is estimated that 40% of proteins have multiple separate roles in the NE as it has been in expected contaminating organelles. In the NE proteome reflect tissue differences is unlikely that the tissue differences observed. In the case of tissues examined and so could not account for the tissue differences observed. In the case of ER and mitochondrial proteins found did not reflect their relative abundance within those organelles. Thus it is likely that the tissue differences observed in the NE proteome reflect tissue differences in expected contaminating organelles. It is in fact more likely that the potential contaminants from these other organelles have separate roles in the NE as it has been estimated that 40% of proteins have multiple subcellular localizations. This idea is supported by the proteomics data in that if the proteins reflected contamination from the other organelles their relative abundance in the NE preparations should reflect their relative abundance within the organelles. However, spectral counts (a semiquantitative measurement of protein abundance used in mass spectrometry based on the number of times a particular peptide is recovered) for the ER and mitochondrial proteins found did not reflect their relative abundance within those organelles. Thus it is likely that many of these potential contaminants are indeed bona fide NE proteins.

Consistent with the likelihood that the identification of the first NE proteins reflected their relatively higher abundance, the NETs that were more tissue-specific were identified with far lower spectral counts than the first characterized ones. Lamin A/C and B1 were identified with 3772 and 2301 spectra respectively and the number of spectra for the first NETs identified were 1826 for LAP1, 1473 for LAP2, 384 for LBR, 751 for SUN1, and 3184 for SUN2. Nurim and emerin were comparatively low with only 51 and 124 spectral counts respectively. In contrast, the vast majority of the new NETs identified and especially those identified in just one tissue had a spectral count lower than 20.

Tissue-Specific Contributions to Disease Pathology

The possibility that some of the newly identified tissue-specific NETs contribute to NE-linked disease pathologies is supported by some having already been linked to other similar inherited diseases (Table 1) and by the pathologies resulting from knockout in mice for others. Lamin A and the well-characterized and similarly widely expressed NETs emerin, nesprin1, nesprin 2, and the soluble α splice variant of the NET LAP2 have all been linked to neuromuscular disorders, muscular dystrophies or cardiomyopathies. In addition the more tissue-specific NETs identified by proteomics of muscle NETs DTNA, VMA21, RYR1 have also been linked to neuromuscular disorders, muscular dystrophies or cardiomyopathies. Thus these NETs could potentially mediate the tissue-specific pathologies of the diseases caused by the widely expressed NETs or potentially cause other variants of these diseases. Other muscle NETs like TMEM38A or Popdc2 are not linked to a disease thus far, but animal models underline their potential role in human muscular disease.
VMA21 was identified only in the muscle NEs and confirmed to target to the NE. Mutations in VMA21—a gene encoding an essential assembly chaperone of the vacuolar ATPase—that result in haploinsufficiency cause a myopathy with excessive autophagy characterized by intracytoplasmic autophagic vacuoles with sarcolemmal features.46 Tmem38A was only identified in the muscle NE proteome, is both very preferentially expressed and abundant in muscle (194 spectra), and is in both the ONM and INM.38,40 Both Tmem38A and its related but more widely expressed homolog Tmem38B appear to function as a counter ion channel for calcium release, which is very important in muscle contraction. Studies knocking out both proteins together resulted in embryonic cardiac failure and dysfunctional skeletal muscle.47 Though less severe, the phenotype of knocking out just the muscle-specific TMEM38A was also focused in muscle, with defects in vascular smooth muscle function resulting in hypertension in intact mice48 and clear evidence of elevated Ca2+ pools in ex vivo muscle with a stronger initial contractile force followed rapidly by muscle fatigue.49 Another protein contributing to this nexus identified in the muscle nuclear membrane and confirmed to target to the NE is the ryanodine receptor 1 (encoded by RYR1) which serves as a calcium release channel. This protein also, unlike most of the tissue-specific NETs, was highly abundant with 235 spectra.38,40 Mice carrying a homozygous targeted mutation in RYR1 died perinatally with skeletal muscle abnormalities.52

<table>
<thead>
<tr>
<th>Protein name</th>
<th>Gene name</th>
<th>OMIM number</th>
<th>Associated disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>VMA21 vacuolar H+ -ATPase homolog</td>
<td>VMA21</td>
<td>610440</td>
<td>Myopathy, X-linked, with excessive autophagy</td>
</tr>
<tr>
<td>RyR1</td>
<td>RYR1</td>
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<td>Lrca</td>
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<tr>
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<tr>
<td>Nesprin 2</td>
<td>SNEC2</td>
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</table>

Table 1. Confirmed NETs associated with disease
**Tissue-Specific Contributions to Cell Cycle Regulation**

The basic regulation of the cell cycle through its stages (G1, S, G2, and M) is common for cells in all tissues. Nonetheless, cells in different tissues distinguish themselves by length, frequency, directionality and cause of induced cell divisions. These parameters can differ for cells in the same tissue or even the same cell type at different stages of differentiation such as in the layers of an epithelium. In fact the best-studied example of a NE link to the cell cycle is a complex that when disrupted in mice causes hyperproliferation of the progenitor layers of epidermis. This complex is formed from lamin A and LAP2α binding together to the retinoblastoma protein (pRb), a key cell cycle regulator. LAP2α is a soluble splice variant of the NE LAP2β. Disruption of this complex resulted in reduced pRb levels and a reduced capacity to undergo cell-cycle arrest in response to DNA damage or accumulation of pRb and G1 arrest depending on the cell type used. These results are consistent with the idea that the Lamin A/LAP2α complex both sequesters and stabilizes pRb so that, depending on the total milieu of cell cycle controls in a particular cell/tissue type, different outcomes can be achieved.

*References*


Though other mechanisms for how NE proteins can influence the cell cycle are less worked-out, such influences are likely relevant to some NE-linked diseases. A lamin A mutant that causes EDMD yielded defects in myogenic differentiation when expressed in a mouse in vitro differentiation system, apparently because myoblasts become unable to exit the cell cycle which is a necessary prerequisite to form myotubes. Additionally, transcriptional fingerprints obtained from both emerin and lamin A-linked EDMD patients revealed that pRb pathways as well as those involving lamin NETs are imprinted suggesting disruption of cell cycle regulation and myogenic differentiation impair muscle regeneration in the disease. This is also consistent with a doubling in the length of the cell cycle observed in tissue culture cells expressing some emerin mutations known to cause EDMD.

One can actually envision many possible ways that NE proteins could affect cell cycle regulation. (1) The first is by direct interaction with cell cycle regulatory proteins such as has already been shown with pRb. In general, sequestration at the NE (and thus away from target genes) of any transcriptional regulator involved in the cell cycle would be expected to have profound effects. (2) A second mechanism by which the NE could influence the cell cycle is involvement in the transmission of signaling cascades from extra or intracellular signals into the nucleus. This could occur via NETs involved in signaling (see signaling section below) or by direct transport through the NPC. (3) Though the NE of higher eukaryotes is disassembled during mitosis, due to the many interactions of lamins and NETs with chromatin, failure to properly break these contacts could activate a checkpoint from lagging/stuck chromosomes (see below). (4) NE proteins can also affect the success of mitosis through separate functions when the NE is disassembled. Lamin B and NPC-associated proteins contribute to spindle function during mitotic chromosome segregation. Tissue-specific NETs may also contribute as a subset of these is enriched at the spindle poles during mitosis (see later cytoskeletal section). (5) Finally, NETs could intersect with various cell cycle regulatory pathways or activate these pathways through mechanical stress or cancer NETs could intersect with various cell cycle regulatory pathways and so on. As noted above, failure to properly disassemble the NE in prophase or reassemble it in telophase could prevent successful mitosis. NE disassembly is driven by phosphorylation of lamins, NETs and nucleoporins to release their associations with chromatin. Failure to fully disengage NE proteins from chromatin could result in blocking of microtubule attachments to kinetochores resulting in lagging chromosomes and aneuploidy. Correspondingly, NE reassembly is driven by dephosphorylation of these NE components; however, unlike disassembly where lamins play a driving role, the NETs and nucleoporins appear to dominate reassembly. This is because many NETs bind chromatin and DNA (reviewed in ref. 92) so that they can direct the membranes in which they are inserted to the mitotic chromosomes. The NET Lap4 (ANKLE2) promotes the dephosphorylation of the chromatin protein BAF by inhibiting BAF’s mitotic kinase NHK-1/Vrk-1 and simultaneously recruiting its phosphatase PP2A. The widely expressed NET Lap2β emerin and MAN1 through binding BAF have been shown to be instrumental in reforming the NE by reinstating chromatin-NE interactions. However, the new tissue-specific NET4/Tmem53 is preferentially expressed in liver, it is possible that it is used for an added layer of cell cycle control that may be needed because liver is both a regenerative organ and the toxin sink for the body and this combination could easily lead to cancer transformation if the cell cycle is not both tightly controlled and responsive to stress. Alternatively, p38 activation could result from NET4/Tmem53 detecting physical stress or mechanical strain during liver regeneration. The FACs-based screen also identified seven other NETs (NET31/Sscpdb, NET3/Tmem209, NET59/Ncln, Trim61, Fam3c, Mag1, Tmem126A; Table 2) with an increased accumulation of 4N (G2/M) cells. Further testing revealed that the effect of all these NETs except for NET59/Ncln was independent of p53, suggesting that they are more likely to be affecting M phase of the cell cycle or operate through a completely novel mechanism. As no significant mitotic abnormalities were observed during the screen, it seems most likely that these effects arrest in G2 in a p53-independent manner opening the exciting possibility that they activate a differentiation program through the regulation of cell cycle exit. This fits with the fact that, like NET4/Tmem53, most of these NETs were very tissue specific and thus might be expected to have tissue-specific cell cycle effects. It is therefore not too surprising that in a separate study NET31/Tmem209 was found to function together with the nucleoporin Nap205 to increase nuclear levels of c-myc and this may explain the role of increased NET3/Tmem209 levels specifically in lung cancers. That roughly 20% of the novel NETs tested had effects on the cell cycle suggests that these types of tissue-specific modifying functions may be quite prevalent.

As noted above, failure to properly disassemble the NE in prophase or reassemble it in telophase could prevent successful mitosis. NE disassembly is driven by phosphorylation of lamins, NETs and nucleoporins to release their associations with chromatin. Failure to completely disengage NE proteins from chromatin could result in blocking of microtubule attachments to kinetochores resulting in lagging chromosomes and aneuploidy. Correspondingly, NE reassembly is driven by dephosphorylation of these NE components; however, unlike disassembly where lamins play a driving role, the NETs and nucleoporins appear to dominate reassembly. This is because many NETs bind chromatin and DNA (reviewed in ref. 92) so that they can direct the membranes in which they are inserted to the mitotic chromosomes. The NET Lap4 (ANKLE2) promotes the dephosphorylation of the chromatin protein BAF by inhibiting BAF’s mitotic kinase NHK-1/Vrk-1 and simultaneously recruiting its phosphatase PP2A. The widely expressed NET Lap2β emerin and MAN1 through binding BAF have been shown to be instrumental in reforming the NE by reinstating chromatin-NE interactions. However, the new tissue-specific
NETs are likely to contribute as much to this process because their nucleoplasmic regions tend to have high isoelectric points for binding the negatively charged DNA and several of these tissue-specific NETs have been found to reposition chromosomes (see later chromosome repositioning section).

**Tissue-Specific Contributions to Signaling**

The NE has to pass signals from a variety of signaling cascades to the genome to trigger changes in gene expression in response to various cellular and extracellular cues. In most cases signals were thought to pass into the nucleus through the NPC by regulated transport of activated transcription factors or other regulators that would activate transcription factors already in the nucleus. However, several widely expressed NETs have now been linked to a handful of signaling pathways. Analysis of changes in transcription profiles between heart from a wild-type or an emerin-null mouse revealed emerin connections to 10 signaling pathways, including Wnt and TGFβ pathways and MAPK and JNK kinase cascades and in skeletal muscle myoD and pRb pathways were affected. One possible explanation of emerin’s effect on a plethora of pathways, as well as on myogenic differentiation, is disruption of miRNA expression in emerin-null cells. Emerin was also shown to bind β-catenin and through it affect the Wnt signaling pathway. This crosstalk between emerin and β-catenin influences adipogenesis so that, though widely expressed, emerin plays significant roles in multiple differentiation pathways. Interestingly, ONM resident nesprin-2 interacts with α-catenin and together they form complexes with emerin and β-catenin. The widely expressed NET MAN1 has separately been shown to affect Smad/BMP/TGFβ signaling. The initial model from this data was that...

<table>
<thead>
<tr>
<th>Table 2. Novel NET functional groupings</th>
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<tbody>
<tr>
<td>NET</td>
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<tr>
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</tr>
<tr>
<td>Cell Cycle</td>
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<tr>
<td>NET4/Tmem53</td>
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<td>NET15/Seyd9</td>
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<tr>
<td>NET31/Tmem209</td>
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<td>Famb1</td>
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<td>Magp1/SIG2</td>
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<tr>
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<td>NET59/Ncln</td>
</tr>
<tr>
<td>NET25/LM22</td>
</tr>
<tr>
<td>NET39/Pucdc3</td>
</tr>
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<td>NET40/Dak</td>
</tr>
<tr>
<td>NET35/Smpd4</td>
</tr>
<tr>
<td>NET38/KAAT161</td>
</tr>
<tr>
<td>Cytoskeleton</td>
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<tr>
<td>NET5/Tmem201</td>
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</tr>
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</tr>
<tr>
<td>WR1</td>
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<td>NET42/TM7SF2</td>
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MAN1 binding to Smads simply sequestered this part of the signaling pathway at the nuclear periphery, away from targets in the genome, thus inhibiting BMP/ TGFβ signaling. However, from recent work it turns out that MAN1 directly competes with the transcription factor FAST1 for binding to Smads and then additionally recruits the phosphatase PPM1A to inactivate the bound Smads, thus delivering a double whammy knockout blow to the signaling pathway. 107 A less direct intersection then additionally recruits the phosphatase PPM1A to inactivate with the transcription factor FAST1 for binding to Smads and so affects TGFβ pathways. 108

Several other NETs identified in the NE proteomics studies have since been linked to various signaling pathways (Table 2). NET25 (Lem2), a paralog of MAN1, was also widely expressed, but was found to be required for efficient myoblast differentiation. NET25 is able to complement emerin’s role in myogenesis, and, like emerin, it negatively regulates the ERK1/2 pathway. 109 NET25 is a member of the LPP family of membrane lipoprotein phosphatases. Its knockdown promoted myogenesis and its overexpression inhibited C2C12 differentiation. 110 NET39 is a member of the LPP family of membrane lipoprotein phosphatases. Its knockdown promoted myogenesis and its overexpression inhibited C2C12 differentiation. 110 NET39 was further found to interact with mTOR and it is thought to act on this signaling pathway in C2C12 myogenesis. 110 Another tissue-specific NET, NET13/SMPD4 is a member of the sphingomyelin degradation pathways. 112 Though far from tissue-specific, NET13/SMPD4 is extremely variable in expression being absent from roughly half of tissues in a large transcriptome study. 63 NET39 is preferentially expressed in muscle and a member of family 31 glycosidases. It is highly expressed in skeletal muscle and upregulated during C2C12 myogenic differentiation. Its knockdown reduces C2C12 differentiation potential by half and its catalytically dead mutant failed to rescue this phenotype, consistent with an important role for this Mdm2-anchored glycosidase in myogenesis. 113 NET39 interacts with the IGF-II precursor and is required for IGF-II secretion. Through this it positively regulates the Akt pathway in C2C12 differentiation. 114 As more novel tissue-specific NETs are tested it seems likely that more such signaling pathways will be uncovered. Notably, unlike most of the original identified NETs, many of these appear to have enzymatic functions to contribute to these processes.

Possible Tissue-Specific Contributions to Cytoskeletal Organization and Mechanical Stability

The intermediate filament lamins and the widely expressed NET SUN and nesprin proteins have been shown to contribute to nuclear mechanical stability in experiments measuring resistance to applied mechanical forces. 115,116 The transmembrane SUN proteins in the INM bind to the lamin polymer via their cytoplasmic tail, which is placed in a nuclease region. This results in a connection to the luminal region of transmembrane nesprins in the ONM. Together this nexus is called the Linkers of the Nucleoskeleton to the Cytoskeleton or LINC complex (Fig. 3). 115 The cytoplasmic regions of most nesprin isoforms include an actin-binding site to connect to the cytoskeletal actin microfilaments. There is also indirect evidence that LINC might be able to also connect to other cytoplasmic filaments as nesprin 3 binds plectin, 116 which can indirectly connect to all cytoplasmic filaments, and microtubule motors have been found to associate with some nesprin isoforms. 117,118 (Fig. 3). Some have also proposed that emerin be considered as an additional LINC complex component. 111 The LINC complex is involved in a number of cellular functions including nuclear positioning, mechanotransduction, cell division and the organization of the cytoskeleton (reviewed in ref. 119). Disruption of LINC complexes induces an overall loss of mechanical stiffness across the cytoskeleton. 120 LINC complex components and possibly the complex itself are additionally important for telomere positioning through SUN proteins 121 and for association of the centrosome with the nuclear membrane through emerin. 122

Although LINC is highly conserved, it is possible that tissue specific proteins interact with the complex and there is even some tissue specificity from orthology and splice variants within the core LINC components. There are five SUN proteins of which SUN1 and 2 are widely expressed while SUN3, 4, and 5 are testis specific. There are four nesprins with many splice variants, and those shown to be involved in EDMD are muscle specific. 123 The nesprin effects in muscle dystrophy could be due to mechanical instability or also to disruption of nuclear positioning under the neuromuscular junction. Both a dominant-negative nesprin mutant and nesprin 1 and 2 double knockout mice fail to recruit synaptenic nuclei to the neuromuscular junction in skeletal muscle. 124,125 In skeletal muscle, levels of nesprin in general are highest in synaptic nuclei, 126 further supporting tissue specificity of function.

Transmembrane Actin-associated Nuclear Lines or TAN lines (Fig. 3) are a subset of actin filaments that direct nuclear positioning in migrating cells through connections that involve LINC complex proteins nesprin2 giant and SUN2. 127 A potential tissue-specific component has recently been added to the TAN lines that in NET5/Samp1 was found to contribute to stabilizing the interaction between the TAN lines, LINC proteins and the nuclear lamina. 128 This interaction is assisted by the fact that NET5/Samp1 has been shown to bind lamin A/C, emerin, SUN1, and SUN2. 127 NET5/Samp1 also may play a role in aspects of cell polarity as its knockdown results in an increase
in the distance between the NE and the centrosome.83 These different aspects of NET5/Samp1 function may be directed in part by different tissue-specific splice forms observed using NET5 antibodies.96 Several NETs identified in the muscle NEs were found in a visual screen to affect aspects of cytoskeletal organization (Table 2).38 Of particular note, two of these muscle NETs appeared to track with microtubules at the nuclear surface, suggesting that there may be another type of LINC complex formed by different NETs that is more specific for microtubule connections. Given the promiscuous interactions of SUN domain proteins and nesprins,120 it is likely that tissue-specific expression of their isoforms as well as potential interactions with tissue-specific NETs, as already shown for NET5/Samp1, may play an important role in the spatial and temporal control of nucleo-cytoskeletal coupling.

**Tissue-Specific Contributions to Genome Organization and Gene Expression**

It has been clearly shown that specific chromosomes, chromosome regions, and chromatin domains have preferred positions in the interphase nucleus and it is thought that this may function to optimize gene regulation. For example, both microscopy and biochemical approaches indicate that the gene-poor (and mostly transcriptionally inactive) chromosomes tend to be at the nuclear periphery while gene-rich (and mostly transcriptionally active) regions locate to the interior (reviewed in128). Though most chromosomes follow this general tendency for gene poor chromosomes to be at the periphery, the spatial organization of genes and chromosomes can also be tissue or cell-type specific. Particular genes or chromosomes tend to be found in the nuclear interior in one cell type while being at the periphery in another (reviewed in ref. 128).

The NE is thought to be one of the major drivers of chromosome and gene positioning within the interphase nucleus due to the fact that mutations in or loss of NE proteins (i.e., lamins and NETs) can result in altered spatial chromosome organization (reviewed in ref. 128). The effects of widely expressed NE proteins on chromosome positioning likely reflects the general tendency for gene poor chromosomes to be at the periphery driven by interactions between lamins and core histones129 and several NETs that bind silenced chromatin. For example LBR binds heterochromatin protein 1 (HP1)130 and LAP2β binds the transcriptional repressor germ-cell less131 and histone deacetylase 3 (HDAC3).132 Accordingly, recent studies have shown these proteins to function in generic aspects of spatial genome organization through an interaction with lamin B1, LAP2β, and HDAC389 and general peripheral positioning of heterochromatin through LBR.134

The mechanism for achieving tissue-specific patterns of spatial genome organization is expected to also function through affinity tethering, but the players have until recently remained obscure. However, with the identification of so many tissue-specific NETs a visual screen was recently engaged that found...
that several extremely tissue-specific NETs could repurpose chromosomes to the nuclear periphery (Table 2).9 Each of these NETs affected at least a subset of chromosomes and different NETs affected different combinations of chromosomes, suggesting that each NET has different binding sites on DNA/chromatin that can function synergistically so that in combination they can achieve a threshold of affinity to move an entire chromosome to the periphery. For example, in liver cells only a liver-specific subset of NETs is expressed (NET45 and NET47 and possibly others). This particular combination of NETs is not expressed in other cell types such as fibroblasts. This results in capturing of a particular chromosome set at the nuclear periphery in liver cells but not in fibroblasts (Fig. 4A and B). As several tissue-specific NETs were also found for other tissues such as muscle, one could imagine that as progenitor cells choose their eventual fate between a muscle and fat cell lineage that changes in NET composition could likewise engineer a particular pattern of spatial genome organization specific to that tissue that could also contribute to its differentiation (Fig. 4C). Though it is not yet known whether these tissue-specific NETs bind DNA, chromatin proteins or transcriptional regulators sitting on particular genes, secondary structure predictions indicate that many have coiled coils or leucine zippers that could be used in interactions with transcriptional regulators and some, such as NET5/Samp1,127 have zinc fingers that could be used for direct binding to specific DNA sequences.

The effects of this repurposing on gene expression are still under investigation, but there are several intuitive mechanisms whereby gene expression could be changed through gene positioning. (1) Local propagation of silencing to new genes recruited to the same general position due to the local abundance of silencing enzymes, for example the affinity of HDAC3 for LAP2β.103 This mechanism could also work in the opposite direction with propagation of activation as the protein originally identified as NET43, now known as hALP1, only has a predicted transmembrane span in certain species, but in humans where it lacks this it can be recruited to the NE through binding to SUN1 at the end of mitosis where it facilitates the decondensation of chromatin.105 (2) Increasing the effective concentration of a particular transcriptional regulator through compartmentalization could also alter gene expression. The NE represents ~1/30th the volume of the nucleus, so LAP2β recruitment of germ-cell less104 could make its local concentration 30-fold higher than the actual concentration in the whole nucleus. Similarly, transcription factors could be sequestered away from targets in the genome as was shown for MAN1 and Smads.104,105 (3) Steric factors blocking access to the DNA for transcriptional activators or alternatively in the structure of tethered chromatin—still unknown—could enable greater access to the local epigenetic silencing enzymes or transcriptional repressors. Testing these various possibilities is hindered by the fact that, when genes move in a physiological context such as differentiation, many additional changes occur within the time frame researchers are able to sample such as the pattern of transcriptional regulators, epigenetic marks on the chromatin, the transcriptional state of the gene, etc. The inability to modulate gene position without additional changes occur within the time frame researchers are able to sample such as the pattern of transcriptional regulators, epigenetic marks on the chromatin, the transcriptional state of the gene, etc. The inability to modulate gene position without additional changes occur within the time frame researchers are able to sample such as the pattern of transcriptional regulators, epigenetic marks on the chromatin, the transcriptional state of the gene, etc.
This discrepancy has generally been attributed to the addition of transport receptors and their substrates that transiently associate with the NPC core structure in the process of translocating through the NPC central channel, but it is also possible that there are as yet unidentified NPC components some of which may be tissue specific.

The first indications of tissue-specificity in the NPC came from observations of tissue-specific expression of transport receptor variants (reviewed in ref. 138). Chief among these is the importin-α/karyopherin-α family, which is encoded by multiple genes that each produces multiple tissue-specific splice variants. Some of these splice variants have been shown to play important developmental roles,139 and this is likely due to their favoring transport of developmentally important nuclear regulators.

It was not long after the first reports of tissue-specific transport receptors that the transmembrane nucleoporin gp210 (also called POM210) was cloned in a study comparing uninduced to induced mesenchyme.140 This was the first demonstration of cell type and developmental specificity in expression of a core component of the NPC. This developmental and tissue-specificity was later confirmed in a wider study in mouse that directly compared it to other nucleoporins POM121 and Nup62 that were expressed in all tissues examined.141 More recently it was found that gp210 is important for both myogenic and neuronal differentiation.142 This followed on work showing that Nup133 is important for neural differentiation in mice143 and that Nup558/RanBP2 also changes during myogenesis.144 This latter study is perhaps the most telling as here it was found that Nup558/RanBP2 levels increase during myogenesis concomitant with a change in the physical architecture of the cytoplasmic filaments of which Nup558/RanBP2 is a primary component.144 This suggests that differences in the physical/mechanical needs of differentiated muscle require either a sturdier cytoplasmic face to the NPC or that a requirement for a higher metabolic load enlists more filaments to capture cargos. Tissue-specific variants of Nup558/RanBP2 and POM210 have also been observed as well as altered expression and splicing for Nup98/96.145-147

It remains to be seen whether any of these new NE proteins are tissue-specific components of the NPC, but it is noteworthy that...
stretches of phenylalanine-glycine (FG) motifs in unstructured regions containing proline are a characteristic feature of many nucleoporins and the NE data sets were strongly enriched for these motifs. Moreover, the third mammalian transmembrane nucleoporin NDC1 was not found in the NPC proteomics, but was identified in the NE proteomics studies. Together these observations argue for at least a reasonable possibility that additional uncharacterized nucleoporins and transport receptors can be found in these data sets.

Potential Tissue-Specific Contributions to Tissue Differentiation and Evolution

Any of the above-discussed functions linked to tissue-specific proteins could have effects on overall tissue differentiation and function. Tissue-specific NETs that play roles in cell cycle regulation could, if defective, reduce the abundance of a particular cell type within a tissue and the ability of the tissue to regenerate when damaged. Defects in signaling, spatial genome organization and gene regulation from tissue-specific NETs could modulate tissue-specific gene expression and the overall metabolism supported by the altered genes. Loss of a tissue-specific NET contributing to cytoskeletal organization could yield defects in the establishment of cell polarity, affecting the efficiency of polarized secretion in the tissue.

In all of the above-mentioned cases, the tissue could conceivably carry defects while retaining the outward appearance of normalcy. The transcription factors driving differentiation and gene expression would still be there, the core of the LINC complex would still provide basic cell mechanical functions, basic nuclear-cytoplasmic transport would still occur. We postulate that the roles of tissue-specific NETs is to fine tune cell functions so that, for example, in some NET-linked diseases loss of an interaction with a tissue-specific NET protein could yield subtle defects that only become pronounced when a muscle is repeatedly stressed of a fat store is called upon to manage a heavier metabolic load. Any athlete can attest that the metabolic and physical loads we normally place on muscle in an average day are minimal compared with the kind of demands placed on the same muscle groups in an athletic competition. Accordingly, the timing of clinical presentation in many NET-linked diseases—when children begin to become more active physically or upon puberty when higher usage, metabolic or endocrine loads would be placed on the tissue—is consistent with this idea of defects in tissue-specific NETs resulting in failure to achieve optimal gene expression or metabolism while initially giving the appearance of normal development.

It is intriguing to speculate that this fine-tuning by tissue-specific NETs helped drive organism complexity. Comparison of the evolutionary conservation of all the NETs identified in the various proteomic analyses revealed that the more tissue-specific NETs were the least evolutionarily conserved. Thus, the tissue-specific NETs evolved as organisms began to distinguish more complex tissues and functions. Interestingly, when searching for orthologs in a wide range of eukaryotes whose genomes have been fully sequenced and annotated, we observed enormous variation even in closely related organisms (Fig. 5).

The red on the heat map indicates no ortholog to the human NET and some human NETs did not even have orthologs in other primates. A sharp drop in the organisms having orthologs of NETs occurs when leaving primates to other mammals with another sharp drop between mammals such as dog, horse and dolphin and mammals such as wallaby, alpaca and platypus (Fig. 5). Yet another sharp drop is observed with birds, and, interestingly, there is more ortholog identity between humans and fish and reptiles than between humans and birds. Very few NET orthologs were found in lower eukaryotes including some of the most commonly used model organisms of worms, flies and yeast (Fig. 5). While these lower eukaryotes are excellent to study the core components of these systems such as the core LINC complex or core NPC, the full functional complexity will only be able to be properly addressed in human tissues. This evolutionary analysis also indicates some interesting outliers that might be worth investing in as model systems to study family complexity. Because of their association in the LINC complex one might think that nesprins would co-evolve with SUN proteins. However, based on ENSEMBL data, in fish nesprins have twice the normal number of orthologs in mammals while SUN proteins have half the number. Chickens on the other hand have jumped from the normal mammalian number of 4–6 SUN protein genes to 36. Understanding what advantage trebling the number of SUN proteins has to chickens when other gene families have not similarly expanded may help to understand fully the functions of this interesting protein family.

The three tissues from which these NET proteomes were determined were chosen because they have widely differing characteristics; however, there is a need for other tissues to be examined. Interestingly, when testing NET expression in various tissues skin was lacking in many of the more widely expressed of the novel NETs.48 We anticipate that there are likely to be large numbers of additional tissue-specific NETs found in skin, brain and germ cells based on this analysis; however, for any tissue linked to disease it makes sense to engage proteomic analyses to identify its most tissue-specific NETs.

Conclusions

Our view of the NE has in the past 20 years evolved from that of little more than a physical wall to a dynamic structure perhaps even more complex than the plasma membrane in its responses in signaling and its variety of functions. Though this review has focused on the more tissue-restricted NETs with less characterized enzymatic functions, there were also many well-characterized membrane proteins identified at the NE that have functions in ion transport, membrane biogenesis, proteolysis and dozens of other functions also relevant to this organelle. In retrospect, this is not surprising when considering that the NE must integrate signals from all over the cell and even from outside the cell to rapidly respond to a wide range of stimuli with changes in gene expression, protein and mRNA degradation, initiation or escape from the cell cycle, nuclear size, nuclear and cell migration, etc. The various tissue NE proteome data sets are rich with information that can be applied to all these functions.
No potential conflicts of interest were disclosed.

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