The nuclear membrane proteome: extending the envelope

The Nuclear Membrane Proteome: Extending the Envelope

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Abstract

The marriage of proteomics with cell biology has produced extensive inventories of the proteins that inhabit several subcellular organelles. Recent proteomic analysis has identified a large number of new putative transmembrane proteins in the nuclear envelope, and transcriptome profiling suggests that the nuclear membrane proteome exhibits some significant variations among different tissues. Cell type-specific differences in the composition of protein subcomplexes of the nuclear envelope, particularly those containing the disease-associated protein lamin A, could yield distinctive functions and so explain the tissue-specificity of a diverse group of nuclear envelope-linked disorders in humans. Considered together, these recent results suggest an unexpected functional complexity at the nuclear envelope.

An introduction to the nuclear envelope

The past few years have seen an explosion in the number of identified nuclear envelope (NE) proteins as well as in the number of their associated diseases. The NE (Figure 1), which is continuous with the more peripheral ER, contains an outer (ONM) and inner nuclear membrane (INM) that are joined at the nuclear pore membrane, giving rise to three different subdomains within nuclear membranes. The ONM is functionally similar to the more peripheral ER, but also is thought to contain some distinct resident proteins. The INM contains a large group of distinctive transmembrane proteins and is lined by a polymer of intermediate filament proteins called lamins. As many as four major lamin subtypes (lamin A, C, B1, and B2) are expressed in mammalian cells in a developmentally-regulated manner. Most lamin subtypes can be modified with lipid moieties that facilitate their targeting to the NE, and a fraction of some lamins occurs in the nucleoplasm as well as at the INM (reviewed in [1]). The lamin polymer and interacting proteins at the INM collectively comprise the nuclear lamina. The nuclear pore membrane is closely associated with nuclear pore complexes (NPCs), the main macromolecular transport channels across the NE. The transport functions and proteome of the NPC have been extensively characterized [2,3], and will not be further considered here. It is generally agreed that the lamina has a role in nuclear morphology and stability [4-6], and support for a wide and ever-increasing array of additional functions has emerged from recent studies. Lamins and lamina-associated membrane proteins have been linked to activities as diverse as transcription [7-10], DNA replication [11,12], nuclear anchoring/ migration within the cell [13], and signaling cascades [14]. To what extent these are direct or indirect effects of lamina proteins remains to be elucidated.

Consistent with the notion that the nuclear lamina is involved in a diverse range of basic cellular functions, at least fifteen inherited diseases and syndromes have been linked to lamins A/C and certain associated NE transmembrane proteins. These include muscular dystrophies, lipodystrophies, neuropathy, cardiomyopathies, dermopathy, bone disorders, and premature aging diseases (reviewed in [15,16], [17,18]). Each of these diseases affects only a limited number of tissues, yet the NE proteins that have been linked to disease – most notably lamins A/C, emerin, LBR, and MAN1 – are expressed widely in differentiated somatic cells. Moreover, different mutations in the same protein can affect different tissues. For example certain mutations in lamins A/C primarily affect striated muscle, while another affects neurons, and others target skin or fat tissue.

It is becoming evident that many NE transmembrane proteins can engage in multiple distinct protein interactions [19]. Moreover, the expression level of these transmembrane proteins can vary between different cell types, as discussed below. This raises the possibility that the tissue-specificity of diseases caused by mutations in widely expressed lamina proteins may be a consequence of the tissue-specific expression patterns of their binding partners. An analogous scenario is found in *C. elegans*, where the NE transmembrane protein UNC83 targets to the NE by an interaction with UNC84 [20]. At early stages of development UNC83 is only observed in tissues where nuclear migration occurs, whereas UNC84 is ubiquitously expressed. Mutations in either of the interacting proteins result in the failure of nuclear migration and the same “<u>unc</u>oordinated” phenotype reflected as loss of worm mobility.

Roads to the nuclear envelope proteome

The pre-proteomics catalogue of mammalian NE proteins was the product of over a quarter century's work utilizing many different approaches. Roughly three decades ago, lamins became the first NE proteins characterized — in large part because they are the most abundant proteins in isolated NEs [21,22]. Subsequently a number of NE transmembrane proteins (Table 1) have come to light, and human diseases have been linked to some of them. LBR (Lamin B Receptor) was identified over a decade after lamins by its binding to lamin B1 [23]. LBR was later found to bind heterochromatin protein 1 (HP1) and DNA as well (reviewed in [24]). LBR is homologous to yeast sterol C-14 reductase and also is enzymatically active. Mutations in LBR cause Pelger-Huet Anomaly [6] and Greenburg skeletal dysplasia [25]. The lamina-associated polypeptides, LAP1C and LAP2ß, were identified using monoclonal antibodies generated against proteins of a NE fraction [26,27]. LAP2ß has been functionally implicated, either directly or through its binding partners, in nuclear growth [28] and transcriptional repression [10]. LAP1 remains poorly characterized, but was recently shown to interact with torsinA — the protein mutated in early-onset torsion dystonia [29]. Nurim (nuclear rim protein) was identified by the screening of a GFP-cDNA fusion library for constructs that targeted to the NE [30]. Emerin was identified genetically as the gene responsible for Emery Dreifuss Muscular Dystrophy [31] two years before its localization to the NE was established by antibody labeling of cells [32]. MAN1 was identified from an autoimmune serum that gave nuclear "rim" staining characteristic of lamins [33]. It has been linked to three related bone disorders: osteopoikilosis, Buschke-Ollendorff syndrome and melorheostosis [18]. Though MAN1- and emerin-linked diseases in humans affect distinct tissues, in *C. elegans* they are synthetically lethal in embryos, suggesting a

common pathway in their functions [34]. The first mammalian member of a new group or proteins, termed Synes (and subsequently also called Nesprins [35]) was identified in a 2-hybrid screen for partners of a kinase of the postsynaptic membrane in muscle: Syne-1 was found to target to the NE using polyclonal antibodies [36]. This family is characterized by variable numbers of spectrin repeats and a conserved domain referred to as a KASH domain (for Klarsicht; [37], Anc-1, Syne-1 homology). Some of the isoforms, which also contain an actin-binding domain, are the largest proteins at the NE and are important for nuclear positioning (reviewed in [38]). UNCL was uncovered in a study to determine why nicotinic acetylcholine receptors do not properly assemble in many mammalian cell lines, and was found to target to the NE by overexpression of a tagged fusion protein [39]. Some of these proteins are thought to be concentrated in the INM and others may be located in the ONM, although decisive immunolocalization to either membrane has not yet been shown for many of them [38]. Two NE transmembrane proteins, gp210 and POM121, were identified as components of the NPC [40,41]. These are the only transmembrane proteins associated with the NPC and are thought to be important for positioning/ tethering of NPCs.

The genomics era has changed both the approaches and the scope in the characterization of new NE proteins. Two genomics-based strategies have recently been used: homology searching and proteomics. The Syne/Nesprin NE protein family was extended when a separate gene (Syne/Nesprin-2) was found in the human genome sequence that had considerable sequence similarity to Syne/Nesprin-1 [36]. Homology searching has also revealed mammalian relatives of proteins originally identified at the NE in worms, and *vice-versa*. For example, two mammalian proteins, Unc84A/Sun1 and SUN2 (Sad-1 and UNC domain), were

found to be homologous to Unc84, a NE transmembrane protein originally identified in *C. elegans* [13]. Both mammalian proteins also target to the NE [42-44]. Conversely, the NE transmembrane proteins emerin and MAN1 were both discovered in mammals, but homology searching revealed *C. elegans* homologs that function at the NE [45]. The LEM domain, originally identified by its presence in 3 NE transmembrane proteins (LAP2-emerin-MAN1), has been observed in other proteins found from proteomic analysis of the NE [46] and from searching of the genomic databases [47]. However, LEM domains are not found exclusively in NE proteins: SANE is a LEM domain-containing protein that functions in the cytoplasm and does not appear in a nuclear fraction [48], and there are LEM domain-containing splice variants of LAP2 without a transmembrane sequence that are concentrated in the nuclear interior [49]. This emphasizes the importance of combining genomic and cell biological approaches to clarify protein localization and function.

The use of proteomics to identify NE-specific transmembrane proteins is hampered by the fact that the ONM is continuous with the peripheral ER and is functionally similar to it (Figure 1). Thus, even though the NE contains specific proteins for its distinctive functions, it also contains proteins present in the peripheral ER. Two different proteomics strategies were used to distinguish NE-specific transmembrane proteins [42,46]. The first study relied on the hypothesis that additional NE proteins would have the same biochemical extraction characteristics as the known transmembrane proteins of the lamina [42]. NEs isolated from cultured neuroblastoma cells were extracted with chaotrope (4 M urea in 0.1 M sodium carbonate), generating an insoluble fraction that is enriched in integral proteins. This was compared to non-ionic detergent-insoluble and salt-insoluble fractions containing most of the known lamina-associated INM proteins (Figure 2A). As the

chaotrope fraction contains both NE and ER transmembrane proteins, it alone is insufficient to distinguish INM proteins. However, proteins of the chaotrope pellet that were also found in the detergent/salt-extracted pellets were considered to be good candidates for novel INM proteins. Each fraction was separated on 2-D gels, and the protein spots were excised and analyzed by MALDI mass spectrometry (Figure 3). This analysis identified most, but not all, previously characterized transmembrane proteins of the INM, as well as mammalian Unc84A/Sun1 and a novel protein with no predicted functions that was named LUMA. Both novel mammalian NE transmembrane proteins were shown to target to the NE by exogenous expression of the proteins fused to GFP [42]. An additional 19 uncharacterized proteins were identified that did not comply fully with the biochemical criteria used. However, as the known NE protein LBR also did not fully satisfy these criteria, some of these may prove to be novel NE proteins.

The second study used a “subtractive” approach to exclude peripheral ER proteins that also were present in the NE fraction [46]. In this case a microsomal membrane (MM) fraction was used to identify integral ER proteins. The MM fraction was analyzed separately from the NE fraction and all proteins appearing in both fractions were subtracted from the NE fraction (Figure 2B). Fractions prepared from rodent liver were analyzed using Multi-dimensional Protein Identification Technology (MudPIT) [50,51], which couples tandem mass spectrometry with multiple liquid chromatography steps to analyze the complex mixture of peptides generated by direct digestion of isolated membranes without prior 2-D gel separation (Figure 3). This method avoids loss of many integral membrane proteins that are poorly resolved on 2-D gels [52]. The subtractive approach was validated by the identification of all expected previously characterized NE transmembrane proteins in

the NE fraction and their absence from the MM fraction. In addition, 67 previously uncharacterized putative NE transmembrane proteins were identified in the NE fraction, which were absent from the MM fraction [46]. To simplify discussion, members of this group were called NETs (for NE Transmembrane proteins). However, they should be considered as "putative" NETs until their localization has been confirmed by a direct assay, involving either targeting of a tagged protein to the NE in transfected cells or immunolocalization. Eight of these NETs were shown to target to the NE when expressed in transfected cells as epitope-tagged constructs. This suggests that many of the remaining 59 also will prove authentic. The large number of novel NE proteins identified in the second study, as compared to the first, is attributable to the high sensitivity of MudPIT (Figure 3) and the fact that the second study analyzed nuclei from liver, which is a composite of many different cell types rather than a cultured cell line.

The "subtractive" and "comparative" approaches used to identify NE-specific proteins both had limitations. The comparative approach disregarded NE-specific proteins that were not associated with the salt- and nonionic detergent-insoluble lamina fraction, because no other basis was provided for distinguishing between the NE and ER transmembrane proteins that were present in the chaotrope-resistant fraction. The subtractive approach disregarded proteins that have functions in both the ER and the NE. For example, a known ER protein, torsinA, appeared in both NE and MM fractions. However, a fraction of torsinA appears to have specific functions at the NE as it is tethered there by interaction with the NE-specific transmembrane protein LAP1 [29]. Moreover, point mutants in torsinA resembling those responsible for the disease early-onset torsion dystonia preferentially accumulate in the NE [53]. This underscores the notion that some integral proteins localized throughout the ER

may have binding sites and functions at the NE different from peripheral ER functions.

Proteomics also has inherent limitations. Protein identifications can be erroneous in the uncommon event that a peptide with a post-translational modification fortuitously has the same mass as a different peptide from a distinct protein. Database inaccuracies also can result in failure to make protein identifications, since database sequences are used to predict the peptide masses used for comparison to the measured masses. Finally, assignment of transmembrane status can be erroneous because most transmembrane prediction algorithms are based on the identification of long hydrophobic α-helices (> ~15-20 residues), yet similar hydrophobic helices also can occur in the interior of folded proteins. Moreover, membrane integration can also occur with multimers of beta-barrels, (reviewed in [54]) and hydrophobic 'domes' inserted into the membrane bilayer as 'monotopic' proteins as postulated for FAAH [55].

Numbering the set of NE proteins

It may be some time before determination of the full set of NE proteins is possible. Aside from the limitations of the subcellular fractionation and proteomics strategies discussed above, there are potential artifacts associated with antibody localization and overexpression of fusion proteins, which can confound a clear determination of NE localization. Most of the previously characterized NE transmembrane proteins, when overexpressed as transfected tagged fusion proteins, accumulate in the peripheral ER as well as in the NE, presumably due to saturation of binding sites at the NE. Moreover, NE targeting can be obstructed by such tags: LAP1C targets normally if GFP is fused at its C-terminus, but not if it is fused at its N-terminus (our

unpublished observations). If some proteins are tissue-specific (see below), a protein may target to the NE only if a relevant binding partner is present; thus ectopic expression in an inappropriate cell type may not yield NE localization. The most rigorous determination of NE localization would involve the use of immunolocalization carried out with multiple affinity-purified antibodies.

Even rigorous testing may be confounded by the existence of multiple splice variants that have distinct cellular locations. Analysis of LAP2 has shown that at least six separate mRNAs are generated from the gene, two of which lack a membrane-spanning segment [56]. In addition to the NE-bound ß-variant, isotype-specific antibodies exist only for the soluble α-variant, which localizes predominantly in the nucleoplasm (with minimal concentration at the nuclear rim) [49]. A wide variety of splice variants is also evident for the Syne/Nesprin protein families [36,38] and for 18 of the 67 new NETs.

At this point, it is unclear how many of the proteins that are localized throughout the ER have distinctive functions at the NE, as suggested for torsinA. There have been many reports of transmembrane proteins that are concentrated at the NE in certain tissues or conditions of cell culture, but that are not specifically concentrated at the NE under other circumstances. These include proteins involved in lipid modification [57], second messenger signaling [58], and peptide/ ion transporters [59,60]. All of the above-mentioned proteins also were observed in the NE-enriched fraction of the “subtractive” proteomics study [46]. Since 566 total proteins were identified in the NE membrane-enriched fraction of that study and 148 proteins were identified in the “comparative” study [42], it is likely that the number of proteins with specific functions at the NE will increase.

Although the focus of this review is on transmembrane proteins, it is noteworthy that proteins lacking a predicted membrane-integrated region also have been found to concentrate at the NE, and many have been shown to bind NE transmembrane proteins or lamins. Among these are several chromatin and chromatin-modifying proteins such as HP1, BAF (barrier-to-autointegration factor), histones, and the transcriptional regulator germ cell-less (GCL) (reviewed in [24]). Several additional soluble RNA and DNA modifying proteins were detected in both proteomics studies. One of these, the mammalian homolog of Dbp5 DEAD box helicase, was subsequently shown to be tethered to the NPC for a role in yeast mRNA export [61]. Such "associated" NE proteins could be parts of larger protein complexes organized around NE transmembrane proteins.

A different NE proteome for different tissues?

UNCL was not identified in the "subtractive" MudPIT analysis of rodent liver NEs [46], consistent with other reports that it is not expressed in liver [39]. This finding raises the possibility that other NETs may have tissue-specific expression. Insight on the expression patterns of most of the NETs identified in the subtractive proteomic analysis comes from data obtained with a large-scale DNA microarray-based study of transcription ("transcriptome" analysis), which involved 61 mouse and 72 human tissues and cell types [62]. For most NETs, considerable variation was observed in expression levels over the tissues sampled. Over 4/5 of those analyzed exhibited greater than a 10-fold range of expression differences among the studied tissues, and many exhibited levels of expression no higher than background in some tissues [63]. Interestingly, some NETs showed preferentially high expression in a small number of tissues, including liver and two of the cell types present in liver, fat

cells and blood (Figure 4). The strongest preferential expression in liver was observed for NET45, which averaged nearly 25-fold higher levels of expression in liver than the median value observed over all tissues. Only a few of the NETs exhibited such extreme tissue-preference. These observations support the hypothesis that each cell type has a small set of unique or preferentially expressed NE proteins. Thus, analysis of NEs from other tissues is predicted to identify some additional NE transmembrane proteins that are absent from liver.

The first indication that NE composition might be dynamic came from observations that lamin subtypes change in their expression during development. Lamins A and C, which are splice variants differing in their C-termini, usually appear at the time of, or following differentiation (reviewed in [1]) whereas lamins B1 and B2 are expressed throughout development, albeit at varying levels [64]. This developmental regulation is likely to be important as it is observed in all vertebrates tested (frogs, birds, mammals) as well as in *Drosophila* (reviewed in [1]). Lamin subtype variation also could influence the composition of membrane proteins in the NE, as several transmembrane proteins have been shown to target to the NE by lamin binding [65]. Although few transmembrane proteins have been tested for lamin subtype preference, LAP2ß appears to have a preference for lamin B1 [66] and emerin for lamin A [67,68]. Similar to lamins, the splice variants of LAP2 have been shown to exhibit dynamic developmental expression [69]; thus, changes in lamina composition during tissue differentiation may direct or facilitate a developmentally coordinated transition of protein complexes at the NE.

Concluding remarks

Surprisingly little is known about the specific functions of NE transmembrane proteins, even those best characterized. Although some enzymatic activities are indicated by regions of homology within some of the new putative NETs, LBR is the only previously characterized NE transmembrane protein that appears to have enzymatic activity (*i.e.* sterol C-14 reductase). Important clues on the functions of certain NE transmembrane proteins has come from analysis of their binding partners. In particular, a number of proteins (*e.g.* emerin, MAN1, LAP2) recently have been found to bind signaling components and/or transcriptional regulators. Emerin binding proteins include lamin A, MAN1, Syne/Nesprin-1α, GCL, BAF, F-actin, myosin I, the Btf transcriptional repressor, and the YT521-B splicing regulator (reviewed in [19]). This may indicate that one of the primary functions of many NE transmembrane proteins is to recruit specific regulatory complexes (such as for chromatin modification or transcriptional regulation) to the nuclear periphery through unique combinations of binding partners. In this way the NE could influence gene expression, and impairment of these functions could lead to disease.

Chromatin regulatory functions could be greatly affected by compositional differences in the NE between different tissues and developmental stages. This encourages further study of the NE from a systems biology perspective. It will be important to sample NE proteomes from a variety of different cell and tissue types in order to determine the extent of NE variation and to identify tissue-specific NE transmembrane proteins. Once additional NETs have been identified and verified, determination of networks of protein interactions will help to discern tissue-specific functional complexes that may be involved in disease.

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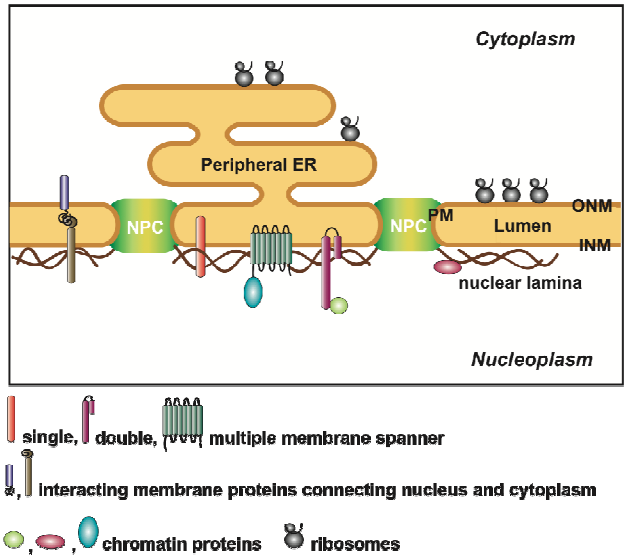
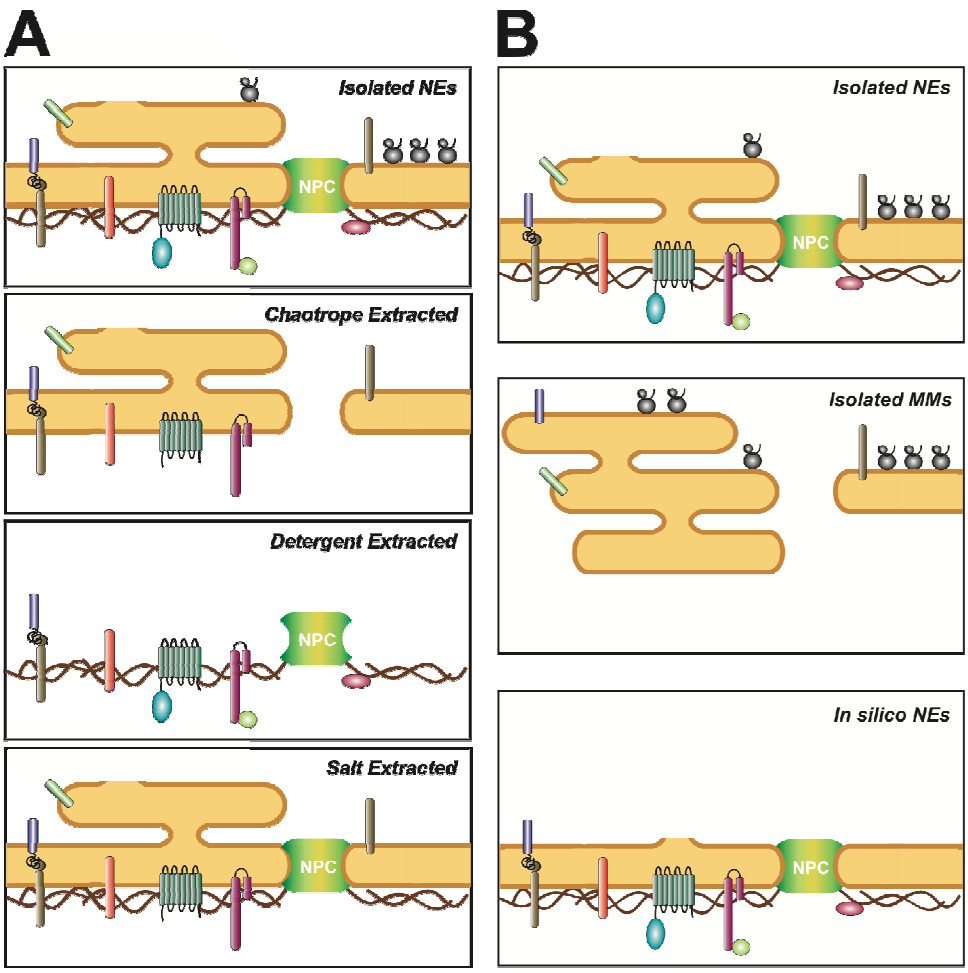
Figure Legends and Tables

Figure 1.



Schematic diagram of the NE. The nuclear envelope consists of outer and inner nuclear membranes connected at the “pore membrane”. The outer nuclear membrane (ONM) is continuous with the rough and smooth endoplasmic reticulum (ER). The inner nuclear membrane (INM) contains many unique integral proteins, which commonly are associated with the intermediate filament lamin polymer. The pore membrane (PM) apposed to the nuclear pore complexes (NPCs) contains specific integral proteins involved in membrane tethering of NPCs. Depending on their topology and membrane subdomain, NE transmembrane proteins could have functions in the cytoplasm, nucleoplasm, or the perinuclear lumenal space. ONM-specific proteins have not been clearly identified, but may include some members of the Syne/Nesprin protein family. Three general groups of transmembrane proteins have been demonstrated in the INM. These include single membrane-spanners with most of the protein mass located in the nucleoplasm (such as LAP2 and emerin), double membrane-spanners that have two nucleoplasmic domains (such as MAN1), and multiple membrane-spanners that have several domains in both nucleoplasmic and lumenal compartments (such as LBR and nurim).

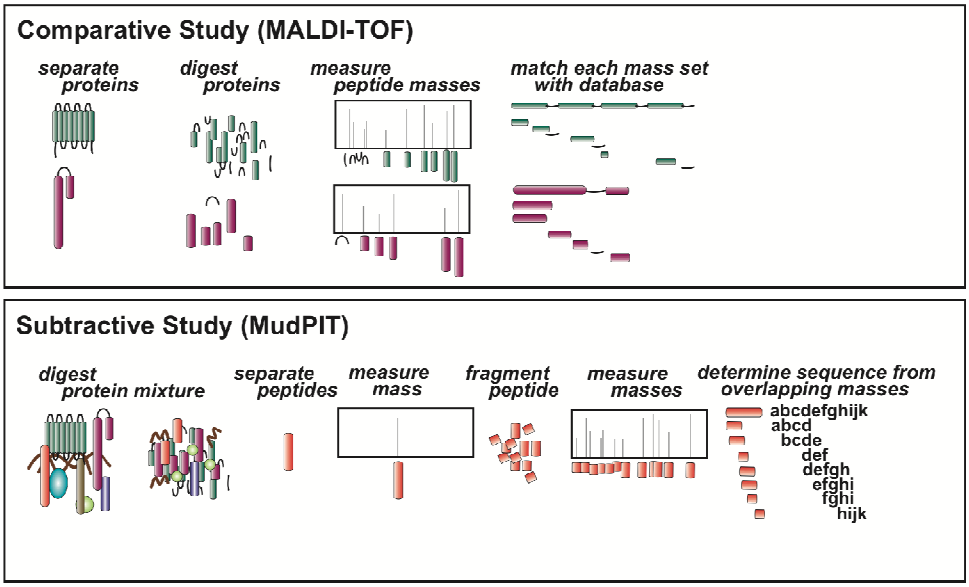
Figure 2.



Proteomics subcellular fractionation strategies. Because of NE continuity with the ER, identification of NE-specific proteins is confounded by ER membranes cofractionating with isolated NEs, and by ER-like functional properties of the ONM.

A. In the comparative approach, NEs were extracted with 4 M urea in 0.1 M Na_2CO_3, generating a chaotrope-insoluble fraction that is enriched in integral proteins. Membrane proteins from both the NE and ER should be well represented in this fraction, but all non-membrane proteins including lamins should be solubilized and extracted. This fraction contained LAP2ß and calnexin, but not lamin B1 or the soluble NPC receptor importin ß. NEs were separately extracted with a non-ionic detergent (1% Triton X-100). This treatment should solubilize membranes and transmembrane proteins that are not tightly bound to a detergent-resistant structure (such as the lamin polymer). Thus membrane proteins associated with the nuclear lamina should appear in this fraction while those of the ER should be extracted. This fraction contained LAP2ß, lamin B1 and importin ß, but not calnexin. The nuclear lamin polymer has unique biochemical properties that make it resistant to high salt concentrations; thus, transmembrane proteins associated with the nuclear lamina should also remain in the salt-insoluble (1 M NaCl) fraction (along with other membrane proteins), while soluble contaminants, such as chromatin proteins, should be extracted. This fraction contained LAP2ß, lamin B1 and calnexin, but not importin ß. B. In the subtractive approach, peripheral ER proteins that also were present in the NE fraction were excluded by discounting proteins that appeared in a separately isolated microsomal membrane (MM) fraction that is rich in ER proteins. Subtracting proteins that appeared in both fractions from the NE fraction yielded an “in silico” NE fraction.

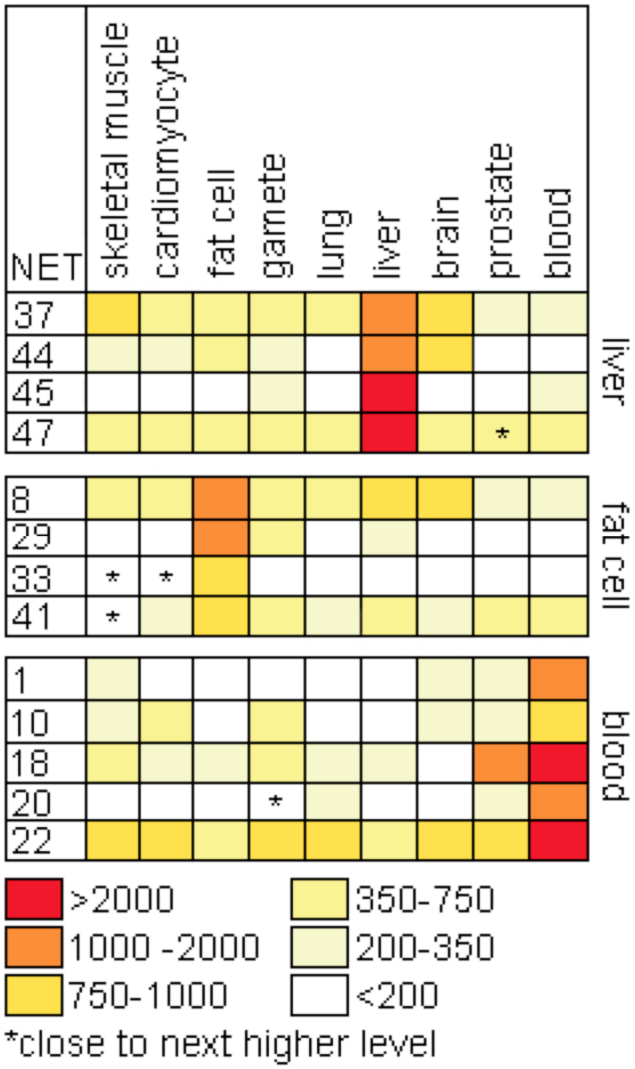
Figure 3.



Proteomics methodologies. Differences in approach for the actual mass spectroscopy method applied between the studies likely also contributed to the differences in the number of protein identifications. In the comparative proteomics

study (upper panel) proteins were first isolated, then digested with specific proteases and the peptide masses measured by MALDI-TOF. Many peptides must be found to match the predicted masses of peptides generated by hypothetical cleavage for a particular protein in the database in order to gain statistical confidence for identification. In contrast, the subtractive proteomics study (lower panel) used MudPIT in which the complex protein mixture is directly digested and multiple liquid chromatography steps are engaged to elute one peptide at a time into the mass analyzer. Once the peptide mass is measured, the peptide is fragmented and the mass of each fragment measured. Computer algorithms relate the possible amino acid compositions that could account for the full-length peptide with those of all the fragments and the known sequences from the protein databases. In many cases this can yield a protein identification from a single peptide.

Figure 4.



Expression diversity of NETs in different tissues. Novel NETs were analyzed for expression levels in different human and mouse tissues using the Novartis transcriptome database accessible at “http://symatlas.gnf.org/SymAtlas/” [62]. Two or more experiments were available for roughly half of the 53 NETs that were represented on the arrays used (of the 59 putative and 8 confirmed NETs). DNA microarray experiments were standardized so that expression levels could be compared between tissues. The values obtained for expression in a subset of tissues were translated into the color-coded schematic shown. Value ranges are listed in the key: a value <200 is considered to be not significantly higher than

background. The NETs listed exhibited considerable tissue-preference in their expression profiles in liver, fat cells or blood. NETs 37, 45, and 47 exhibited the highest expression levels in liver of all the tissues and cell types analyzed. NET44 expression levels in liver were slightly superseded in amygdala, but were still 4-fold higher than the median expression level. Both adipocytes and brown fat are considered together as “fat cells”. In the cases of NETs 8, 33, and 41, expression in liver is also high (3rd, 3rd, and 4th highest levels of expression respectively in individual experiments).

Table 1. NE-specific transmembrane proteins tested for NE targeting

protein	resistance to detergent suggesting lamina association	shared domains	evidence for alternate splice variants	tissues affected in disease	year identified	reference
LBR	yes			neutrophils bone	1988	[23]
LAP1	yes		+		1988	[26]
gp210	ND				1990	[40]
LAP2	yes	LEM	+		1993	[27]
POM121	ND				1993	[41]
emerin	yes	LEM		muscle	1996	[32]
MAN1	yes	LEM		bone	1996	[33]
nurim	yes				1999	[30]
Syne-1	ND	Spectrin repeats KASH	+		2000	[36]
Syne-2	ND	Spectrin repeats KASH	+		2000	[36]
UNCL	yes				2000	[39]

Unc84A	yes	SUN			2001	[42]
LUMA	yes				2001	[42]
SUN2	yes	SUN			2002	[43]
NET3	yes				2003	[46]
NET4	no				2003	[46]
NET8	yes		+		2003	[46]
NET26	no				2003	[46]
NET31	no		+		2003	[46]
NET39	yes				2003	[46]
NET51	yes				2003	[46]
NET56	yes		+		2003	[46]

ND, not determined

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