Nuclear networking

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ABSTRACT
Nuclear lamins are intermediate filament proteins that represent important structural components of metazoan nuclear envelopes (NEs). By combining proteomics and superresolution microscopy, we recently reported that both A- and B-type nuclear lamins form spatially distinct filament networks at the nuclear periphery of mouse fibroblasts. In particular, A-type lamins exhibit differential association with nuclear pore complexes (NPCs). Our studies reveal that the nuclear lamina network in mammalian somatic cells is less ordered and more complex than that of amphibian oocytes, the only other system in which the lamina has been visualized at high resolution. In addition, the NPC component Tpr likely links NPCs to the A-type lamin network, an association that appears to be regulated by C-terminal modification of various A-type lamin isoforms. Many questions remain, however, concerning the structure and assembly of lamin filaments, as well as with their mode of association with other nuclear components such as peripheral chromatin.

KEYWORDS
lamin; laminopathy; nuclear envelope (NE); nuclear pore complex (NPC); superresolution microscopy

The nuclear envelope (NE) is, by definition, a structure that is unique to eukaryotes, forming the interface between the nucleus and cytoplasm. Accordingly, it is the NE that imposes both the spatial and temporal segregation of nuclear versus cytoplasmic processes, and in this way furnishes regulatory possibilities that are unavailable to prokaryotes. The general structure of NE has been highly conserved through evolution. Its most prominent features are 2 lipid bilayers, the inner and outer nuclear membranes (INM, facing the nucleoplasm; ONM, facing the cytoplasm), which are separated by a ~50 nm gap or perinuclear space (PNS). Although biochemically quite distinct, the INM and ONM are actually connected at numerous annular junctions that form aqueous channels between the nucleus and cytoplasm. These channels are occupied by nuclear pore complexes (NPCs), massive multiprotein assemblies that regulate the back and forth movement of macromolecules across the NE.

As with the NE as a whole, NPCs are also highly conserved. Electron microscopic studies have revealed a core structure displaying eightfold radial symmetry. This consists of a central framework positioned at the level of the nuclear membranes that embraces a central gated channel. The cytoplasmic aspect of the central framework features 8 flexible 100–150 nm filaments. By contrast the nucleoplasmic face anchors a “basket-like” structure that extends about 100 nm into the nucleoplasm. Extensive proteomic and genetic studies have identified roughly 30 NPC subunit proteins or nucleoporins, with each present in multiple copies. Complementary ultrastructural analyses have mapped all of these nucleoporins, several of which are transmembrane proteins, to discrete subdomains within each NPC.

Metazoan NEs possess an additional structural element, the nuclear lamina. In thin section transmission electron micrographs, the lamina appears as an amorphous layer 10–30 nm thick that is intimately associated with both the nuclear surface of the INM and the underlying chromatin. Although conventionally defined as an integral component of the NE, in certain regards the lamina may be thought of as a transitional structure that contributes to both NE and chromatin organization.

The nuclear lamina is composed primarily of type V intermediate filament (IF) proteins known as nuclear lamins. These proteins first emerged in coelenterates (such as hydra) as the products of a single gene that represents the ancestor of the entire IF gene family.
family. Both birds and mammals possess a repertoire of 3 lamin genes encoding proteins that fall into 2 distinct sequence classes, A-type and B-type. As a general rule, B-type lamins are constitutively expressed and can be detected in all nucleated cell types.7-9 The A-type lamins, by contrast, are absent from early embryonic cells, and even in adults these proteins cannot be detected in some stem cell populations. In humans, as well as in other mammals, the single A-type lamin gene (LMNA) encodes 2 abundant proteins, lamin A and lamin C, as well as 2 relatively minor isoforms, lamin AΔ10 and lamin C2.6 While lamins A, C and AΔ10 are found in somatic cells, lamin C2 is expressed only during spermiogenesis. Two prominent somatic cell B-type lamins, B1 and B2 are encoded by LMNB1 and LMNB2 genes respectively. A third B-type lamin, Lamin B3, is derived from LMNB2 by alternative splicing, and is found exclusively in male germ line cells.

The structure of both A- and B-type lamins conforms to the same basic scheme that is observed in all IF proteins.10,11 This comprises a small N-terminal head domain, a central α-helical coiled-coil and a globular C-terminal tail domain. In the case of the lamins this latter feature is organized about an immunoglobulin fold.12,13 The lamins are also unique among IF proteins in that they contain a nuclear localization sequence (NLS) just downstream of the coiled-coil. The NLS is essential for appropriate targeting of newly synthesized lamins to the nucleus. Both lamin A and the B-type lamins have at their C-termini a CaaX motif, where C is cysteine, a is an aliphatic amino acid and X is usually methionine or leucine.14 This motif undergoes a series of posttranslational modifications, commencing very soon after synthesis and before incorporation of the parent molecule into the nuclear lamina.15 The first step involves farnesylation of the cysteine residue via the formation of a thioether bond. Farnesylation is followed by proteolytic cleavage of the aaX residues. In the case of LaA, this is catalyzed by a metalloproteinase Zmpste24. By contrast, in the B-type lamins aaX cleavage is mediated by RCE1. Finally, the newly exposed C-terminus is carboxy methylated.16 Once incorporated into the nuclear lamina, LaA, but not the B-type lamins, is subjected to a second proteolytic cleavage also by Zmpste24. This results in the elimination of 15 residues from the LaA C-terminus, including the farnesylated cysteine. Thus in contrast to B-type lamins, farnesylation of LaA is only transient. The presence or absence of the farnesyl lipid tail has significant implications in terms of how A- vs. B-type lamins interact with the INM.

A number of human diseases, collectively known as laminopathies, are associated with defects in lamin proteins.17-19 In particular, different mutations in LMNA have been variously linked to forms of muscular dystrophy, cardiomyopathy, lipodystrophy and premature aging syndromes (progeria). Several of these are characterized at the cellular level by altered lamin distribution and abnormalities in nuclear and NE architecture. These diseases have highlighted the role played by the nuclear lamina in the maintenance of NE integrity as well as in defining global nuclear organization. Recent studies have revealed that cells and tissues that are subjected to high mechanical stresses display increased expression levels of LMNA.20 Indeed, in cells migrating through confined spaces, LMNA expression helps to prevent NE breakage. However, upregulation of LMNA also leads to increased nuclear stiffness and reduced deformability. This may limit the ability of cells to pass through the smallest apertures.21,22 In this way, modulation of LMNA expression allows the cell to strike a balance between nuclear deformability and nuclear fragility.23

It is clear that the function of the nuclear lamins in defining nuclear mechanics is intimately linked to the structure of the lamina. Early studies on purified rat liver NEs revealed the lamina to be highly insoluble and most likely filamentous in nature.24,25 These findings were congruent with the later identification of the A- and B-type lamins as IF proteins. Additional purification steps facilitated the isolation of a membrane-free NPC-lamina fraction suggesting a close and stable association between these 2 distinct NE structures.25 The first detailed visualization of the nuclear lamina layer was that of manually isolated *Xenopus laevis* oocyte NEs.26 Low-angle rotary shadowing combined with transmission electron microscopy,26 as well as later studies based on high resolution scanning EM methods,27 revealed a meshwork of intermediate-like filaments with associated NPCs. In places, parallel and orthogonal filament arrays were evident with cross-over spacings of about 50 nm. The giant oocyte nucleus is particularly amenable for such analyses since it contains little chromatin to obscure the nuclear face of the NE. However, while providing unique and valuable insight into the organization of
the nuclear lamina, amphibian oocyte NEs are unusual in that the lamina is composed primarily of a single B-type lamin (lamin LIII). This obviously raises the issue of whether a somatic cell lamina, containing 3 or 4 lamin species, would be organized in a similar fashion. Unfortunately the ultrastructural techniques that have been so useful in the oocyte, cannot be applied to somatic cell nuclei. Because of the extensive association of chromatin with the nuclear face of the NE it has not proved possible to obtain en face views of the somatic cell nuclear lamina.

While direct visualization of the somatic cell lamina has proved challenging, the behavior of isolated lamins has provided some useful insight to lamina organization. Purified worm, chicken and rat lamins form ~50 nm rod-shaped homo-dimers in vitro. These will spontaneously assemble into IF-like filaments, roughly 10 nm in diameter, and laterally associate to form paracrystals with a ~25 nm banding pattern. While these studies are entirely consistent with a filament based lamina, how such filaments might be combined at the INM remained uncertain.

Recently, Goldman and colleagues using 3-dimensional structured illumination microscopy (3D-SIM) have mapped all of the major mammalian lamin isoforms, lamin A, C, B1, and B2, at the nuclear periphery of mouse embryonic fibroblasts. Each of the lamins appears to assemble into a meshwork extending over the entire surface of the INM, consistent with their proposed roles in providing mechanical integrity to the NE. Importantly, changes in the remaining lamin meshwork were found, at the micron scale, in cells deficient for either A- or B-type lamins, suggesting potential interactions between the different lamin meshworks. However, questions remain whether these interactions are characteristic of individual lamin molecules, or the collective meshworks. To answer these questions, more detailed characteristics within the meshwork in terms of possible filament branching, segmentation and association with other components of the NE at the sub 100 nm level, are needed.

With the help of both direct stochastic optical reconstruction microscopy (dSTORM) and photoactivated localization microscopy (PALM), related techniques of superresolution (SR) single molecule localization microscopy, our group was able to visualize the nuclear lamina in situ in mouse adult fibroblasts. With a lateral resolution of ~40–50 nm, the

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**Figure 1.** Exogenously expressed LaA forms a filamentous network at the nuclear envelope. (A) Lmna-/- MAFs expressing exogenous mEos2-LaA protein were fixed, and subjected to PALM super-resolution imaging with the microscope focused at the surface of the nucleus. This was followed by EMST network analysis, showing mEos2-LaA localizations (red) and its computed network (magenta). (B) Top: area highlighted in (A) was enlarged, showing strings of mEos2-LaA localizations (red); bottom: computed mEos2-LaA network (magenta) in the same enlarged area.
lamina appeared as an extensive anastomosing mesh of filament (Fig. 1). Computational reconstruction suggested that lamin filaments are organized as overlapping yet independent networks formed by homo-
oligomers of lamin A, C and B1. This is consistent with earlier observations, showing that A- and B-type lamins form either separate structures or are segregated in to separate domains within a single structure. Due to additional limitations in vertical res-
olution, none of the SR techniques used here are able to define parameters such as filament thickness, or to determine whether lamin filaments associate in to bundles. It was also not possible to determine whether filament branching contributes to the appearance of the lamina network. These issues will ultimately need to be addressed in future studies, potentially using EM tomography. Nevertheless, when taken together, these data support the notion that, as in amphibian oocytes, the mammalian somatic cell lamina is formed as a network of filaments. On the other hand, the somatic cell lamina seems to be completely lacking in the regular-
ity that is so clearly evident in its simpler oocyte counterpart. A gratifying aspect of the work is that computational reduction in resolution of the PALM and dSTORM derived images reproduces the results of Shimi et al. In this way we are able to confirm and extend the original structured illumination data.

Given its location at the interface between the INM and underlying chromatin, it is no surprise that the lamins are associated with both integral proteins of the INM as well as soluble nucleoplasmic components. Indeed appropriate localization of certain INM proteins is known to be dependent upon association with nuclear lamins. The interactions of A-type lamins in particular, have been the object of intense scrutiny. This is a reflection of the fact that mutations within the LMNA gene are known to cause at least a dozen tissue-specific diseases. Since LMNA is expressed in virtually all adult tissues, the various LMNA-linked disease phenotypes are likely a reflec-
tion, in part, of altered associations with lamin inter-
actors that are themselves expressed in a tissue specific manner. The 2 major A-type lamins, A and C, derived from LMNA by alternative splicing, differ only in their C-terminal regions downstream of the Ig fold that forms the core of the C-terminal domain. Lamin A contains a unique sequence of 96 residues terminating in the CaaX motif. This region of the mole-
ecule appears to be relatively unstructured. Lamin C, in contrast, contains a unique C-terminal peptide of only 6 amino acid residues and has no CaaX motif.

We have used BioID analysis, an in vivo proximity-
dependent biotinylation assay to identify potential A-
type lamin-associating factors. The majority of these (~100) displayed comparable associations with both major A-type lamins. However, a select group of 20 nucleoplasmic proteins were found to preferentially associate with lamin C. These included transcriptional regulators, histone modifiers, DEAD box helicases, splicing and replication factors, as well as Tpr, a con-
stitutive component of the basket structure on the nucleoplasmic face of NPCs. Only a single protein, SUN1, an integral protein of the INM, was found to show a preferential association with lamin A,

Figure 2. NPCs are clustered in the absence of A-type lamins. *Lmna*+/+ and *Lmna*−/− MAFs were fixed and labeled with the NPC-specific antibody QE5 (green), and subjected to dSTORM super-resolution imaging with the microscope focused at the nuclear surface.
consistent with earlier observations. The close association between NPCs and the nuclear lamina has been known for over 40 y. Moreover, in Lmna-/- mouse fibroblasts NPCs tend to form clusters in the NE (Fig. 2). In comparison in wild type fibroblasts, NPCs are uniformly distributed across the nuclear surface. These observations would suggest that A-type lamins associate either directly or indirectly with NPCs and are essential for their normal distribution.

The discovery of Tpr as an A-type lamin-associated protein provides a potential link between NPCs and the nuclear lamina. Support for this notion was provided by a combination of PALM and dSTORM showing that NPCs preferentially associate with lamin C-containing networks (Fig. 3A and B).

How might one rationalize the difference in association with Tpr of lamin C vs. lamin A? An obvious possibility is that Tpr specifically interacts with the unique C-terminal hexapeptide of lamin C. However, this is unlikely to be the case. The reason for this is that a constitutively farnesylated lamin A mutant (L647R) shows the same preferential association with NPCs as lamin C (Fig. 3C). Our conjecture is that the Tpr/NPC binding site is located on the Ig fold region of the lamin A/C tail domain. Access to this site may be restricted by the unstructured post-Ig fold sequence of mature lamin A, but not by the much shorter post-Ig fold sequence of lamin C. In the case of lamin A L647R the post-Ig fold sequence may be constrained in a more “open” conformation through interaction of the farnesyl lipid tail with the INM. In this way the Tpr/NPC interaction site may be exposed in both lamin C and lamin A L647R, but not in wild-type mature lamin A.

These models of lamina organization and association may provide insight in to the molecular etiology of the various laminopathies. For instance, Hutchinson-Gilford progeria syndrome (HGPS) is most frequently caused by a single mutated LMNA allele in which activation of a cryptic splice site results in the production of a permanently farnesylated form of lamin A termed lamin AΔ50 or progerin. HGPS patients begin to manifest features of accelerated aging as early as 1 y after birth and rarely survive beyond their mid-teens. Mortality is almost invariably due to cardiovascular disease. Progerin behaves in a dominant fashion. Both HGPS patient fibroblasts as well as cells expressing recombinant progerin, display nuclear structural abnormalities including nuclear shape changes and heterochromatin redistribution. In addition progerin expression is associated with increased DNA damage and premature senescence. Most significantly, progerin expression is associated with changes in NPC function. This is manifest as a

Figure 3. Exogenously expressed LaA and LaC form filamentous networks at the nuclear envelope, and exhibit distinct associations with NPCs. Lmna-/- MAFs expressing exogenous mEos2-tagged LaA (A) and LaC (B), respectively, were fixed and labeled with the NPC-specific antibody QE5, and subjected to sequential dSTORM (green) and PALM (red) super-resolution imaging with the microscope focused at the nuclear surface.
reduction in nuclear concentration of RanGTP, a small Ras-related GTPase that controls the directionality of nuclear transport across their NE. At the same time, progerin expression leads to loss of Tpr from NPCs.9

The implication here is that progerin may exhibit enhanced association with Tpr. Indeed given the results with lamin C and lamin A L647R, this is precisely what one would predict. Clearly the super resolution methods combined with interactome analyses may together shed new light on the organization and interactions of the nuclear lamina in both health and disease.

In summary, the work described in this study provides an expanded view of the nuclear lamina in mammalian somatic cells. It is entirely consistent with the notion that the lamina represents a composite structure comprised of independent yet overlapping A- and B-type lamin filament networks. It is clear that mammalian nuclear lamina appears more complex than its amphibian oocyte counterpart, but at the same time lacks any ordered filament arrays. Left unanswered, however, is the nature of the A- and B-type lamin filaments themselves. Do these resemble cytoplasmic IFs or are they specially tailored to their unique environment at the chromatin-INM interface? A related question concerns how farnesylated lamins are organized into filaments and how this will affect the interactions of the lamin filaments themselves with the INM. It would appear self-evident that not all farnesylated B-type lamins within a single lamin filament can have equivalent interactions with the INM. Similarly, we have little insight in to the differential association of chromatin domains with A- vs. B-type lamin filaments. At least some of these issues may be resolved through the application of EM-tomography. The use of BioID in the study has provided molecular insight into the mechanisms by which NPCs are anchored to the nuclear lamina, an observation first recorded 40 y ago. Certainly different A-type lamins may strongly influence NPC distribution. However, certain cell types, including early embryonic cells, lack A-type lamins. Therefore it seems highly likely that additional associations between the nuclear lamina and NPCs have yet to be uncovered. Indeed, Nup153, a highly mobile nucleoporin and NPC nuclear basket component, has been implicated in interactions with the nuclear lamina.

Disclosure of potential conflicts of interest
No potential conflicts of interest were disclosed.

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