Phosphorylation of lamins determine their structural properties and signaling functions

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Lamin A/C is part of the nuclear lamina, a meshwork of intermediate filaments underlying the inner nuclear membrane. The lamin network is anchoring a complex set of structural and linker proteins and is either directly or through partner proteins also associated or interacting with a number of signaling protein and transcription factors. During mitosis the nuclear lamina is dissociated by well established phosphorylation-dependent mechanisms. A-type lamins are, however, also phosphorylated during interphase. A recent study identified 20 interphase phosphorylation sites on lamin A/C and explored their functions related to lamin dynamics; movements, localization and solubility. Here we discuss these findings in the light of lamin functions in health and disease.

Lamins are Intermediate Filament Proteins

Intermediate filaments (IFs) comprise a diverse protein family including more than 70 genes, giving rise to an even higher number of proteins, as they often are alternatively spliced.1 The IFs share a common structure consisting of a structurally conserved rod domain flanked by variable head and tail regions.2 Except for the lamins all IFs are cytoplasmic proteins that are part of the cytoskeleton. In mammals the nuclear IFs are the lamin A (LMNA) gene products lamin A, lamin A Δ10, lamin C and lamin C2 as well as the lamin B1 (LMNB1) and lamin B2 (LMNB2) gene products lamin B1, lamin B2, and the germ cell specific splice variant lamin B3.3,4 In the nucleus the lamins make up the lamina, a thin, fibrous layer inside the inner nuclear membrane, but they are also found in a soluble form inside the nucleoplasm.5,7 The location and solubility of lamins affect their functions.

IFs are found in all metazoan organisms,8 with a variable degree of complexity in the expression patterns. While the more simple organisms express only lamins, the complexity of IFs increase in higher order organisms, with the exception of arthropods, which mostly seem to lack cytoplasmic IFs.9 Invertebrates have in general only one B-type lamin whereas A-type lamins only exist in vertebrates, indicating that lamin A is an evolutionary more recent protein.10 The cytoplasmic IFs found in lower organisms are more closely related to lamins than to cytoplasmic IFs in higher organisms.11 This indicates that a B-type lamin was the first IF to evolve and that this founder IF gradually gave rise to cytoplasmic IFs and later to A-type lamins.

A-and B-Type Lamins

The B-type lamins are found in virtually all cells regardless of their differentiation state. In C. elegans deletion of its single lamin leads to lethality early during development.15 Surprisingly, B-type lamins are not required for murine embryogenesis, but mice lacking them die at birth.12-14 However, mouse embryonic stem cells lacking all lamins are able to proliferate and differentiate, demonstrating the tissue specificity in lamin functions.16 The exact role for each lamin, therefore, still remains to be elucidated.
Phosphorylation of N-terminal S22 and C-terminal S392 in human lamin A/C or analogous residues in B-type lamins (often referred to as "mitotic sites") have been shown to induce the disassembly of the nuclear lamina. Phosphorylation of the mitotic N-terminal serine (S22 in human lamin A) appears to be more important for lamin head-to-tail de-polymerization, than the C-terminal site.28,29

The N-terminal phosphorylation, however, is not considered sufficient enough to cause complete disassembly of the lamina in vivo and Cdk1 might be working collectively with other mitotic related kinase(s) to facilitate this depolymerisation. In fact, PKC has been identified as an additional kinase required for lamin B1 disassembly through lipid signaling.30

Also Cdk5 has been shown to phosphorylate the mitotic sites in the context of neuronal apoptosis,31 and S404 has been shown to be an AKT-target.32 Given the numerous identified phosphorylation sites and the variety of contexts during which phosphorylation have been described to take place it is likely that additional kinases still remain to be discovered.

Few of the many identified lamin A/C phosphosites have been studied in detail, as a significant part of the data originates from high-throughput mass-spectrometrical analyses. These analyses have significant limitations, as the sites are typically not validated and there is no information on the relative stoichiometry of these sites. Especially the role of phosphorylation of lamin A during interphase has not been thoroughly investigated. We recently conducted a study in which we identify 20 phosphorylation sites from interphase cells and in detail investigate the structural function of a few of them using single site mutations and advanced imaging techniques.33

Phosphorylation of lamin A/C during interphase

In interphase cells we found that the phosphorylation of lamin A/C is concentrated to 3 distinct regions, the head, the beginning of the tail, and the end of the tail.33 (Fig. 1) As some of these sites have been previously identified as mitotic sites, cell synchronization by mitotic shake-off, and a phosphospecific antibody against S22 were employed to validate that this site is phosphorylated also during interphase. Using Western blotting and immunofluorescence, we could quantitatively demonstrate that S22 is indeed phosphorylated in interphase but, as expected, at a much lower rate than during mitosis.

To study the function of the interphase phosphorylation sites in detail, we utilized GFP-tagged lamin A vectors with mutations of the most occupied phosphorylation sites identified. Substitution of serine for aspartic acid was used to mimic phosphorylation and substitution of serine to alanine as to mimic a phosphorylation-deficient state.

By live cell imaging, we showed that the phosphomimetic mutations of S22, S392 and the double site S404, S407 lead to an increase in nucleoplasmic lamin A and, conversely, phosphodeficient mutations of S390 and S392 lead to increased lamina associated lamin A. The effects of different combinations of phosphomimetic and phosphodeficient mutations of both S22 and S392 showed that phosphorylation of S22 is dominant over S392 and that the double phosphorylation has an additive effect on the localization.

Apart from regulating the localization of lamin A (lamina vs. nucleoplasma), the phosphorylation of S22 and S392 also influences the intranuclear mobility of lamin A, both in the nucleoplasm and in the lamina, as shown by FRAP and FCS. The phosphorylation facilitates faster movements, the functions of which may be associated with the roles of lamin A in the control of signaling and transcription (see the section below). The cellular in vivo observations on increased mobility and dynamics, were reflected in the biochemical properties of the protein, as it was obvious that phosphorylation also affected the solubility of lamin A. Also in this case the phosphomimetic S22 and S392 mutants were more soluble than the wild type lamin A both in the lamina and in the nucleoplasm and S22 was dominant over S392.

Interestingly, the combined phosphomimetic substitution of S22, S392 and S628 lead to lamin A being found in the...
cytoplasm in a portion of the cells. S628 is missing from progerin, the splice variant of lamin A, which is expressed in the devastating early aging laminopathy Hutchinson–Gilford progeria syndrome. This finding implies that this phosphorylation site has a physiological role in the compartmentalization of lamin A. Conversely, the absence of the site in progerin could be one of the mechanisms underlying the progeria phenotype. Lamin B2 has recently been reported to have an important role in the cytoplasm where it interacts with mitochondria promoting their function. Although a cytoplasmic role for lamin A still remains to be described, the fact that phosphorylation could induce a cytoplasmic shift of lamins indicates the possibility of such cytoplasmic function(s).

**Physiological Relevance of Phosphorylation of Lamin A/C**

Recently, lamins have emerged as potential mechanosensors that receive information and respond to changes in the structural and mechanical properties of cellular substrates and surroundings. A recent study showed that cells grown on softer substrates have softer nuclei and, conversely, that cells grown on stiffer substrates have stiffer nuclei. The changes in nuclear stiffness were related to the levels of lamin A. Interestingly, also phosphorylation of lamin A was affected by substrate stiffness. Apart from lamin A levels, the phosphorylation by itself could be a key factor in determining nuclear stiffness. In light of the study described in the preceding section, the phosphorylation induced by changes in substrate stiffness will also be consequential for the sequestration, dynamics, mobility, and transport of lamin A. These aspects may, in turn, be consequential for the signaling and transcriptional properties of lamin A. Lamin A interacts with and regulates chromatin organization and lamins also directly and indirectly influence transcription by regulating transcription factors. The phosphorylation of lamin A is thus a requirement to ensure both successful 3D-migration and survival. Although nothing is known about lamin A phosphorylation during migration, it is tempting to speculate that the increased mobility of phosphorylated lamin A could be one way promote nuclear deformability and thereby facilitate migration. As lamin A has been reported to be both up- and down-regulated in different types of cancers, growth and cell cycle promoting kinase activity could be a way for the cell to influence nuclear stiffness and promote migration.

Although the nucleoplasmic pool of lamin A has been studied for more than 2 decades, the functions of this pool and what regulates it is still barely known. Regarding its potential signaling functions, eluded to above, lamin A interacts with lamina-associated polypeptide 2 isoform α (LAP2α) in the nucleoplasm and this interaction regulates the activity of retinoblastoma protein (pRb) and cell cycle progression. In the absence of LAP2α, nucleoplasmic lamin A is lost, indicating that LAP2α stabilizes or sequesters the nucleoplasmic lamin A. Our results, showing that phosphorylation is important for the shuttling of lamin A between lamina and nucleoplasm, offer a new paradigm to understand the mechanisms that drive the lamin A LAP2α interactions. Interestingly, the lamin region interacting with LAP2α is the beginning of the tail and the Ig-fold, a region where we identified numerous phosphorylation sites that could potentially affect the binding between lamin A and LAP2α. Thus, phosphorylation could influence both the localization, the opportunity to bind, and the direct binding of LAP2α and lamin A.

**Lessons from Laminopathies**

To this day almost 20 different diseases or syndromes caused by over 450 different mutations in the *LMNA* gene have been described. These diseases, collectively referred to as laminopathies, are characterized by their diverse but very specific phenotypes, ranging from lipodystrophy, affecting the fat deposition in certain parts of the body, to muscular dystrophies with or without cardiac involvement, to cardiomyopathies affecting either the left or the right ventricle. How mutations in the very same gene of a protein that is expressed in almost all differentiated cell types can give...
rise to organ and tissue-specific diseases remains a mystery. The possible connection between phosphorylation and laminopathies has not been studied. However, there are a few cases which highlight the potential role of this post-translational modification in diseases. It has been reported that the total phosphorylation of lamin A is lower in muscle biopsies from Emery-Dreifuss muscular dystrophy (EDMD) patients compared to healthy controls but not which sites are affected or what impact this has on the disease. In contrast, S458 has been reported to be specifically phosphorylated in muscle biopsies from myopathy patients with mutations in the Ig-fold of lamin A but not from patients with other lamin A mutations or healthy controls. The authors speculate that mutations in the Ig-fold opens the lamin structure and exposes S458 to Akt1 which was shown to target this site. Our identification of phosphorylation of the same site from interphase HeLa cells suggests that the phosphorylation also have a non-pathological role and that this modification is regulated in a cell specific manner. Lamin A R453W, the mutation that gives rise to the above-mentioned S458 phosphorylation, is one of many muscular dystrophy-associated mutations. Interestingly, this specific mutant lamin has also been shown to have significantly lower S390 phosphorylation compared to wild type lamin A. This shows that even distant mutations can affect phosphorylation status of the whole protein, but we cannot explain why. The decreased phosphorylation can be due to the mutation changing the assembly properties and/or the mechanical or structural properties of lamin A. The decreased phosphorylation can also change the mechanical or structural properties of lamin A, which could be a way for the cell to compensate for the mutation. The phosphorylation states of different phosphorylation sites can also possibly affect the phosphorylation of others. One site could inhibit or promote the modification of another site, for example, by affecting the assembly properties and/or the conformation of the protein. Whether the change in lamin A phosphorylation in myopathies is a result of the mutations or a compensatory mechanism still remains to be clarified.

Laminopathy mutations could also directly affect the phosphorylation of individual sites. For example, recently, a mutation of P4R (proline at 4 to arginine) of lamin was reported. This mutation gives rise to a mild progeroid phenotype, including tight, hyperpigmented skin and lipodystrophy. P4 is located between T3 and S5, both sites phosphorylated during interphase. This mutation disrupts the TP- motif, thereby most likely leading to a deregulation of T4-phosphorylation. The charged side chain of proline is also likely to influence the phosphorylation of S5.

Interestingly, a case of a heterozygous S22L mutation has been reported. This patient suffered from dilated cardiomyopathy with premature ventricular beats requiring a heart transplant. However, no molecular data from the case is available. Bearing in mind that the lamin A phosphorylation sites are highly conserved, reflecting low tolerance for mutations, it is not surprising that so few of the large number of reported lamin A mutations directly target known phosphorylation sites.

Conclusions

We propose that the interphase-specific phosphorylation of lamin A provides additional means to regulate the protein function in a cell and tissue specific manner.
The identification and validation of interphase-specific in vivo phosphorylation sites on lamin A/C and characterizing the role of these in distribution and dynamics of lamin A is just the beginning of understanding what could be the possible functions of these sites. Given the many diverse functions of lamin A/C, also phosphorylation sites which are seemingly not giving rise to major phenotypes in terms of distribution or dynamics can still have important roles with major impact on the role of these in distribution and dynamics in vivo phase-specific.

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