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ABSTRACT

Although its properties have long been used for both typing and prognosis of various tumors, the nuclear envelope (NE) itself and its potential roles in tumorigenesis are only beginning to be understood. Historically viewed as merely a protective barrier, the nuclear envelope is now linked to a wide range of functions. Nuclear membrane proteins connect the nucleus to the cytoskeleton on one side and to chromatin on the other. Several newly identified nuclear envelope functions associated with these connections intersect with cancer pathways. For example, the nuclear envelope could affect genome stability by tethering chromatin. Some nuclear envelope proteins affect cell cycle regulation by directly binding to the master regulator pRb, others by interacting with TGF-ß and Smad signaling cascades, and others by affecting the mitotic spindle. Finally, the NE directly affects cytoskeletal organization and can also influence cell migration in metastasis. In this review we discuss the link between the nuclear envelope and cellular defects that are common in cancer cells, and we show that NE proteins are often aberrantly expressed in tumors. The NE represents a potential reservoir of diagnostic and prognostic markers in cancer.

Keywords: nuclear envelope, lamins, NETs, cancer, genome stability

1. Introduction

The nuclear envelope (NE) is a double membrane system that includes the nuclear lamina plus hundreds of transmembrane proteins, generally called NETs (for NE transmembrane protein), which work together to generate an enormous complexity of functions. This functional complexity is further enabled by compartmentalization within this organelle that can segregate proteins to outer, inner, and pore membranes, the lumen, and the underlying lamina (Fig. 1). The outer nuclear membrane is continuous with the endoplasmic reticulum [1] and contains several NETs that connect the nucleus to cytoplasmic filament systems and the centriole, lending to potential contributing roles in cell polarity and mobility. These NETs connect across the ~50 nm spacing of the NE lumen to inner nuclear membrane NETs that in turn connect to the intermediate filament lamin polymer — the nucleoskeleton that lines the surface of the inner nuclear membrane (reviewed in [2]). The lamin polymer and its bound NETs are collectively referred to as the nuclear lamina. Many lamina components bind chromatin and so may influence gene regulation and genome stability (reviewed in [3, 4]). Nuclear pore complexes (NPCs) that direct trafficking of proteins in and out of the nucleus are inserted in areas where the outer and inner membranes bend to meet at what is called the pore membrane [5-7]. NPCs can affect gene regulation because they are the only ports of entry for gene regulatory proteins and also because some NPC proteins have additional functions in the nucleoplasm during interphase [8, 9].

Lamins have been experimentally linked to functions ranging from nuclear shape and stability [10-13] to replication [14-17], transcription [18, 19], and splicing [19]. Many NETs have also been linked to nuclear shape [20-23] and transcriptional regulation [24-27] as well as signaling cascades [28-30]. As it seems unlikely that lamins and NETs alone could have the wide range of disparate functions attributed to them, it has been suggested that many NE proteins may serve as a scaffold onto which functional complexes assemble.

Most of the hundreds of NETs were only recently identified in proteomic studies; so very little or nothing is known about them. However, the proteomic data lend to two striking observations. First, many NETs are highly tissue specific [31, 32]. Second, Gene Ontology (GO) analysis of proteins identified at the NE that have previously known functions, indicates that a large proportion of NE proteins function in signaling and gene regulation [31]. This suggests that the NE serves, like the

plasma membrane, as a major signaling node of the cell. Adding this to the many functions experimentally linked to the NE, it is likely that there are many separate mechanisms through which NE dysfunction could result in tumorigenesis.

2. History of lamin loss and nuclear shape/volume as prognostic indicators in cancer

Observations of nuclear size and shape variations in tumor cells can be traced back at least as far as the work of Lionel S. Beale at Kings College London in 1860 [33] and continue to be used today. One of the major contributors to nuclear shape and size are the lamin proteins that make up the intermediate filament polymer underlying the NE. Soon after lamins were identified in the 1970s loss of A-type nuclear lamin proteins began to also be used as a prognostic indicator for various tumors [34, 35].

2.1 Nuclear lamina composition changes in development, differentiation and tumorigenesis

The four major lamin proteins (A, C, B1, B2) tend to be expressed in most differentiated tissues at relative ratios characteristic to each tissue [36]. Lamin A is not detectable in most of early embryonic development when cells are highly pluripotent and rapidly dividing. It begins to accumulate in some differentiating tissues at embryonic day 8 in mice [37], but is not observed in the simple epithelia of lung and liver until well after birth [38]. Lamin A remains absent from most blood cell lineages, but it becomes induced as macrophages develop [36, 39, 40]. Induction of differentiation in monoblastoid cells activated expression of A/C lamins and vimentin, a cytoplasmic intermediate filament protein [41]. This was accompanied by reduction of cell proliferation and stronger substratum adherence. However, upon removal of the inducer in the culture medium the differentiated cells lost this differentiation, reduced their adherence and began to proliferate, in what was termed “retrodifferentiation”. At the same time the levels of A/C lamins and vimentin were strongly reduced [41]. These observations led to the hypothesis that A/C lamins might somehow ‘stabilize’ differentiation an idea supported by the finding that lamin A was often absent from tumors and one theory for the rapid division of tumor cells at the time was that they had lost their differentiation [42-44]

As the number of tumor types for which A/C lamin levels were measured increased, it became clear that “retrodifferentiation” was insufficient to account for all observations as many tumors were found where higher A/C lamin levels correlated with more aggressive tumors. For example, small and non-small lung cell carcinoma cells could readily be distinguished by differences in A/C lamin levels [43]. If the v-RasH oncogene was introduced into a small lung cell carcinoma line the A/C lamin levels increased while it gained more aggressive tumor characteristics [43, 45]. Similarly, in nodular sclerosing Hodgkin’s disease lamin A was co-expressed in some cells with Ki67 in contrast with non-diseased reactive lymph nodes where the staining was mutually exclusive [46]. This clearly linked cell proliferation with the lamin A expressing cells. As well as the levels of lamin A/C, its localisation is often abnormal in tumors. For instance, nuclear aggregates of lamin A/C and aberrant cytoplasmic staining has been observed in gastrointestinal and lung carcinomas [47, 48]. Thus, though A/C lamin levels remain a valid prognostic marker for individual tumor types, for some types increased levels are associated with better clinical outcomes while for other types the opposite is true.

Neoplasm associated changes in other lamins have also been reported. As well as reduced lamin A/C levels, lamin B1 was reduced in colon carcinomas and adenomas, and gastric cancers [47]. In addition, hyperphosphorylation of lamin B2 has been reported in leukemia [49]. The structure and function of the nuclear lamina is regulated by phosphorylation, as it causes depolymerization of lamins at metaphase [50, 51]. Thus, changes in lamin phosphorylation, and not just their expression levels, can have a profound effect on the structure of the NE. Phosphorylation could also affect lamin interactions with signaling proteins that are discussed later in section 5.

2.2 Nuclear volume and shape control by NE proteins

Cells of a wide range of sizes, from yeast to mammalian, maintain a roughly constant ratio of nuclear to cell volume, the karyoplasmic ratio [52, 53]. This is a controlled process as nuclear transplantation of a small hen erythrocyte nucleus into a larger cell results in an increase in the size of the small nucleus [54]. Though it is still not clear how the karyoplasmic ratio is regulated, particular karyoplasmic ratios appear to be characteristic of cell type and differentiation stage. Measurements of the karyoplasmic ratio in epidermal layers revealed that nuclear volume was largely maintained, but cytoplasmic volume increased four fold as cells passed from basal to granular layers [55]. A similar distribution in nuclear volume was also observed in lymphocytes between resting and proliferating cells [56]. This suggested that the

characteristic ratios observed in various tumors might reflect the stage of the progenitor cell that originated the tumor, and could be used as prognostic markers.

By the 1970s nuclear size was commonly used as a prognostic indicator for various tumors [57, 58]. For example urinary bladder carcinomas were distinguished on a I-IV grade scale with an extremely wide range in nuclear volume observed [59]. Another study that found a correlation between invasiveness of bladder carcinomas and nuclear volume additionally looked at NPC density. Interestingly the more aggressively behaving tumors also had a higher NPC density [60]. Nuclear volume increases were also correlated with more malignant lesions using an experimentally induced system for oral carcinoma in hamster cheek pouches [61]. However, following these observations outside of a particular specialty can be somewhat confusing as different tumors had different effects of nuclear size [62]. For example, statistically significant associations were observed between larger nuclear volumes and malignancy for invasive meningiomas and bladder carcinoma [63, 64], but smaller nuclear volumes correlated with malignancy for squamous cell carcinoma of the lung [65].

This led to the supposition that nuclear volume might be regulated by the cell cycle; however, inhibition of cyclin-dependent kinases does not affect nuclear growth, indicating that other proteins are involved [66], Recently NE proteins have been linked to the control of nuclear volume [67], although most of them have limiting functions that do not necessarily reflect a specific regulatory role. For example, mutations in NPC proteins (e.g. gp210 [68]) would reduce protein import into the nucleus while disruption of vesicle fusion (e.g. p47 [69]) would be generally limiting for membrane growth.

The NET LAP2β may have a more regulatory role in nuclear growth. A fragment of LAP2β dominantly blocked nuclear volume increases that occur during S-phase in both HeLa cells [70] and an in vitro *Xenopus* system [71]. According to the BioGPS transcriptome database LAP2β is upregulated to >3x its median expression value in tumor cells from colorectal adenocarcinoma, ovarian carcinoma, lung small cell carcinoma, burkitts (Daudi) lymphoma, follicular lymphoma and chronic myelogenous leukemia among others [72-74]. Thus its action may underlie some of the nuclear size increases observed in tumors.

The relationship between nuclear volume control and control of the karyoplasmic ratio still needs to be directly addressed. It is also not clear how nuclear size changes regulate nuclear functions. One possibility is that the changes in molecular crowding as the nucleus enlarges during differentiation and tumorigenesis

affects the formation of macromolecular machines involved in gene transcription and RNA splicing [75].

Nuclear shape changes are also associated with a variety of cancers. Invaginations of the NE are observed in a wide variety of cancer types whereas the segregation of the nucleus into many lobes or polylobulation is principally observed in adenocarcinomas [76]. Grooves or clefts along the nucleus are observed in papillary thyroid carcinoma, urothelial tumors, granulosa-cell tumor of the ovary, and follicular lymphomas [76].

As the major structural proteins of the nucleus [11, 13, 77] lamins likely underlie such shape defects. Interestingly, most lamin subtypes are farnesylated. This addition of short chain lipids onto proteins helps associate them with membranes and is commonly observed on small GTPases linked to cancer such as Ras [78]. The lipid moiety added to lamin A is normally transient and when the farnesylated form accumulates it results in nuclear shape defects and inhibition of cell division, without nuclear size effects [78, 79]. Farnesyltransferase inhibitors have been tested as anti-tumor agents and appear to have some efficacy in correcting the nuclear shape defects, though their anti-tumor efficacy is more likely due to effects on small GTPases. Nonetheless, the abundance of lamins make them a good pharmacodynamic marker for the drug effectiveness in inhibiting the lipid modification [80].

3. The contribution of peripheral chromatin connections to genome stability

The lamin polymer confers stability to the NE and therefore provides a good tethering point for chromatin in the nucleus. In theory lamin A binding to chromatin could hold chromosome territories in place, reducing the likelihood of chromosome translocations or general chromosome breakage. This was supported by the observation that mutating the caspase cleavage site in lamin A delayed the DNA fragmentation that occurs in apoptosis [81]. The absence of lamin A in tumors derived from tissues where lamin A is normally present thus led to the hypothesis that lamins might stabilize the genome.

3.1 Structural support from an elastic nucleoskeleton

Lamins are the only known filament system in the nucleus and their loss or mutation yields defects in nuclear morphology [11, 13, 77]. Lamins have very different properties from other filament systems. Actin and tubulin assemble like bricks and rigidly maintain their structure to a certain pressure, but then fracture when this is exceeded (~35 dyne/cm^2 and 20% strain for actin and 7 dyne/cm^2 and 70% strain for tubulin). In contrast, intermediate filaments stretch and do not break even at 60 dyne/cm^2 and 90% strain [82]. This is because coiled-coil dimers first assemble head-to-tail thin filaments that then bundle so that adjacent coiled-coils from different thin filaments interact in the final 32 molecules-in-cross-section 10 nm filament [83]. Individual thin filaments can thus move relative to one another to stretch the fiber when force is applied.

An elastic NE may actually be a wise strategy for protecting the genome. The nucleus has to withstand considerable forces from the inside, as it grows roughly four-fold during interphase due to import of transcription machinery and genome replication. It also has to withstand very strong forces from the cytoskeleton pushing and pulling as cells crawl when migrating. The NE has many connections (Fig. 1): between the ONM and the cytoskeleton [84, 85], between the ONM and INM via the LINC (linker of nucleoskeleton and cytoskeleton) complex [86], between INM proteins and the lamin polymer (reviewed in [87]), and between both INM proteins and the lamin polymer and chromatin (reviewed in [3]). The sum of these connections throughout the NE enable a strong interface that can both resist and adapt to forces exerted on it. Proteins lost from ruptures of the plasma membrane can be remade, but a chromosome lost from breakage of the NE cannot be recovered. Thus the NE is only likely to rupture in a regulated process such as autophagy.

Despite its elasticity, the NE is nonetheless one of the most stable structures in the nucleus and, as such, provides a framework on which to organize the spatial distribution of the genome. This can have many benefits. For example, tethering of chromosomes to the periphery can keep the chromosome territories separate from one another to reduce the probability of translocation events. NE association could also sterically block access to regulatory proteins. These various outcomes would be determined by the specific interactions between the chromatin fiber and particular NE proteins. Accordingly, a wide range of specific interactions between the NE and chromatin proteins has been described.

3.2 General chromatin-NE interactions

Lamins interact with specific regions of chromosomes called Matrix or Scaffold Attachment Regions (MARs and SARs; [88, 89]), the minor groove of single-

stranded DNA [89, 90], and histones H2A and H2B [91, 92]. The direct interactions with DNA are likely due to low affinity binding of the coiled coil rod [93] as the cytoplasmic intermediate filament protein vimentin could also interact with DNA [94]. In contrast, the interaction with histones has a much higher affinity [91, 92]. Nonetheless, as there are ~3,000,000 [95] copies of lamins in a mammalian cell nucleus, the additive effect of such a large number of low affinity interactions should be highly relevant. Human chromosomes associate with the nuclear lamina over more than 1,300 sharply defined regions ranging between 0.1 and 10 Mb in size called LADs (lamina associated domains) [96]. Genes contained within LADs are generally repressed [96, 97]. Investigation of LAD dynamics in a mouse ES cell differentiation model revealed that lamina-chromatin associations change progressively as differentiation proceeds, frequently involving regions that contain genes associated with a particular differentiation profile [97]. Genes that dissociate from the lamina often become immediately active, others remain inactive but are unlocked for activation at a later stage in differentiation [97]. The repressive environment of LADs at the NE appears to be epigenetically regulated, involving histone acetylation and H3K9 methylation (reviewed in [98]). In this context it is interesting to note that H3K9 methylation has been found to be distributed in discrete blocks of up to 5 Mb, highly conserved in human and mouse genomes, called large organized chromatin K9 modifications (LOCKs) [99, 100]. LOCKs comprise about 4% of the mouse genome in undifferentiated ES cells, but up to 31% in differentiated ES cells and even higher in some terminally differentiated tissues, and may represent a mechanism to lock gene expression down as cells differentiate [99]. The behavior of LADs during tumorigenesis has not been directly addressed yet, however LADs and LOCKs show a remarkable overlap, with over 80% of LADs being contained within LOCKs [100], and LOCKs are substantially lost in human cancer cell lines [99]. It is tempting to suggest that cancer cells would probably exhibit a LAD pattern resembling a more undifferentiated state from the tissue of origin, with H3K9 methylation loss from LADs, inducing a more stem-cell like phenotype.

Although much less abundant than lamins, NETs are likely to play an important role in tethering chromatin to the NE. While only a small proportion of NETs have been tested, nearly all of these have specific associations with different chromatin proteins (Fig. 2). The lamin B receptor (LBR) binds histones H3/H4 [101] and heterochromatin protein 1 (HP1) α and γ [102]. LBR also interacts with the methyl-CpG binding protein MeCP2, an important epigenetic regulator associated with cancer [103-106]. LAP2β binds to barrier-to-autointegration factor (BAF) [107,

108] that plays a role in the folding of chromatin into higher order fibers [109]. LAP2β also binds histone deacetylase 3 (HDAC3; [27]) and the transcriptional repressor germ cell-less (gcl; [26]). In fact several NETs and NPC proteins can bind various transcriptional regulators [25, 110-112]. NPC protein associations with chromatin are complex. While in yeast NPCs associate with boundary/insulator activity that links them to both active and inactive chromatin regions [110, 113-115], in mammalian cells several nucleoporins have separate populations and functions at the NPC and in the nucleoplasm. Those in the nucleoplasm tend to activate transcription while those at the periphery tend to repress transcription [8, 9]. Consistent with this, ChIP of chromatin associated with NPCs indicated a strong favoring of silencing marks [116]. Similar to the NPCs ChIP experiments on chromatin associated with the NET LBR have identified mostly repressive marks [117].

The use of epigenetic marks enables rapid and transient changes to the landscape of the NE, thus dynamically regulating the interaction between NE and chromatin. For example, HP1 and LBR associate through core histones H3/H4 in an acetylation dependent manner. Hyperacetylation of H3/H4 using CREB-binding protein (CBP) *in vitro* abolishes the interaction of LBR with HP1 [101]. Similarly, the association can be abolished *in vivo* by using histone deacetylase inhibitors [118].

The epigenetics of cancer have been the subject of extensive investigation. DNA methylation and histone modifications in particular play important roles in tumorigenesis (reviewed in [119]). The emerging links between the NE and epigenetic factors such as MeCP2, HDAC3, and HP1 strongly suggests that the NE could mediate cancer-related epigenetic changes, perhaps contributing to the silencing of tumor suppressor genes. Regardless, disruption of gene expression patterns by altering the epigenetic landscape could easily contribute to tumor generation.

3.3 NE establishment of tissue-specific chromosome positioning patterns reflective of translocations

Individual chromosomes have characteristic positioning patterns with respect to the nuclear periphery. This correlates partly with gene density e.g. human chromosome 19 is very gene rich and is generally located in the nuclear interior while chromosome 18 is gene poor and tends to be at the periphery [120]. However, other chromosomes tend to be at the periphery only in certain tissues, for example in mouse chromosome 5 tends to be internal in liver and blood cells while being peripheral in lung [121].

The observation that chromosomes have non-random patterns of localization specific for particular tissues suggests that certain chromosomes may be more likely to be adjacent one another in some tissues, but not in others. Genes located in different chromosomes have been shown to interact, using chromosome conformation capture techniques [122, 123], particularly in studies of grouped genes functioning as transcription factories [124-126]. Investigation of experimentally induced double-stranded breaks (DSBs) and translocation events in mouse B cells has shown that interchromosomal translocations occur frequently, and that they are more likely to occur near transcription start sites [127]. Therefore the contact between two DSBs on different chromosomes, prior to the establishment of a translocation event, may have its origin in the mechanics of transcription. Investigation of chromosome positions in normal and cancer cells using whole chromosome fluorescence in situ hybridization (FISH) revealed that chromosomes most commonly involved in translocation events, for a particular tissue, tended to be positioned adjacent to one another during interphase in that tissue [128]. Thus tissue-specific patterns of interphase chromosome positioning, in addition to tissue-specific transcriptional programs, can create a template for the tumor-type specific translocations that drive many cancers via specific interactions between chromatin and the NE.

3.4 NE association with telomeres and recombination

In addition to NE protein interactions with DNA and specific chromatin proteins there are also interactions with specific structural or functional subdomains on chromosomes. Some of these are cell type specific: for example all centromeres are at the NE in human neutrophils [129] while all telomeres are at the NE in sperm cells [130]. However both centromeres and telomeres are distributed throughout the nucleus in most cell types.

The telomere association with the NE in spermatocytes is called the meiotic bouquet, where telomeres align at one side of the nucleus. In mammalian cell types that do not maintain telomere associations with the NE in interphase, the meiotic bouquet tends to be established in early prophase before NE breakdown. It has been suggested that its primary function may be to organize chromosome pairing for homologous recombination [131, 132]. The association with the NE skeleton could certainly provide advantages for properly lining up chromosomes and this would minimize the possibility of translocations during meiotic recombination. A particular class of NETs, the SUN domain proteins, is critical for achieving this configuration. SUN3 is particularly interesting as it is predominantly expressed in testes. Knockout

of Sad1 in yeast [133, 134] or SUN2 in rat [130] weakened telomere associations with the NE. Thus NET mutations in spermatocytes could result in weaker associations between homologous chromosomes, increasing the probability of chromosome mispairing leading to a higher frequency of translocations and consequently tumors.

4. The NE in DNA damage repair

Double-stranded DNA breaks (DSBs) may arise from external sources, for example gamma rays and UV-light, and from internal ones such as free radicals and errors in DNA processing. The regions flanking DSBs are rapidly marked by the core histone H2 variant gamma-H2AX [135] which facilitates the assembly of DNA repair factors. The two mechanisms of DNA repair in mammalian cells are "homologous recombination repair" that requires the template of a sister chromatid and "non-homologous end joining" (NHEJ).

In NHEJ, the broken ends are bound and thus marked by a protein heterodimer, Ku70/Ku80, and are subsequently processed and ligated by a phosphorylation-dependent mechanism (reviewed in [136]). This mechanism is active throughout the cell cycle and simply restores the integrity of a DNA molecule by joining its ends. It is error prone, and can often introduce deletions into the repaired locus as well as generate chromosome translocations. Despite these safeguards, sometimes a DSB escapes repair. This can lead to growth arrest at the G_2/M DNA damage checkpoint. However in yeast the checkpoint was overridden after about 15 hours [137]. Persistent DSBs are relocated to the NE and this process requires a NET, the SUN domain protein Mps3p [138]. Another NE protein, the nucleoporin Nup84, is also required for the tethering of DSBs to the NE [139, 140].

It is not clear why persistent DSBs are relocated to the NE. Perhaps this is a backup system that allows the recruitment of DNA repair factors more effectively or the association of DSBs with the NE might stabilize the genome sufficiently to overcome a checkpoint. Regardless, there is clearly an active mechanism that recognizes broken chromosome ends and relocates them to the nuclear periphery. Correspondingly, it seems that this or another mechanism can also relocate the NE to an internal DSB as treatment with etoposide, which induces DSBs [141], results in lamin B1-containing invaginations of the membrane into the interior of the nucleus. Thus, the NE might contribute to maintaining genome stability by tethering

unrepaired DSBs in a transcriptionally repressive environment that reduces their probability of joining to create a translocation event (discussed in section 3.3) as seen often in tumor cells.

5. Intersection of cell signaling proteins with the nuclear membrane

Lamins and NETs appear to participate in signaling pathways by binding to transcription factors and sequestering them away from their targets. Upon activation of the signaling pathway the transcription factors would be released so they can then act on their target genes. For example, lamin A binds the retinoblastoma protein (pRb) [142], a master cell cycle regulator, and the oncoprotein c-fos [143]. The abundance of lamins makes them ideal to sequester transcriptional regulators. Lamins could exert this function from either NE or separate nucleoplasmic pools [144]. In contrast the NETs are embedded in the membrane, so all interactions with transcriptional regulators and signaling cascades are necessarily restricted to the NE. The NET MAN1 interacts with multiple Smads, part of the TGFβ/bone morphogenic protein (BMP) signaling pathway. MAN1 has an antagonistic effect in this pathway, affecting the phosphorylation of R-Smads, which results in their export to the cytoplasm and attenuating the signaling [29, 30]. Defects in MAN1 cause the bone disorders osteopoikilosis and melorheostosis [145, 146]. Another interesting example is that of the NET emerin, which actually negatively regulates itself. The transcription factor Lmo7 activates emerin gene expression, but at the same time it can also bind to the NET, which sequesters Lmo7 at the NE away from the gene in a negative feedback loop [25]. Emerin is also linked to the β-catenin/Wnt signaling cascade. Emerin binds β-catenin, both sequestering it at the periphery and promoting its export [28]. Both the Smad and β-catenin/Wnt signaling pathways affected by MAN1 and emerin are misregulated in a number of tumors [147-149]. In addition, another NET antagonist of the TGFβ pathway, dullard/NET56, is overexpressed in many tumors [72-74]. While many NET interactions with regulatory proteins keep them away from their targets, other NET binding partners function at the NE. The first such interaction identified was the binding of LAP2β to germ cell-less (gcl), a repressor of E2F/DP transcription factors. Overexpression of LAP2β in cultured cells inhibited E2F-dependent transcription from a reporter construct [26], presumably by recruiting gcl to

act on genes at the nuclear periphery. Another transcriptional repressor with a different target specificity that binds emerin is Btf, a cell death-promoting factor [112]. Thus, the nuclear lamina and other proteins in the NE are able to affect important signalling pathways that are often abnormally regulated in tumors.

6. Nuclear envelope protein involvement in cell cycle regulation

Lamin A binds the cell cycle master regulator/tumor suppressor pRb [142] and the apoptosis regulator/tumor suppressor protein E1B/p19 [150]. In addition, several NETs have recently been linked to the regulation of the cell cycle [151]. This indicates a wider range of mechanisms through which the NE may contribute to cancer.

6.1 Cell cycle misregulation in nuclear envelopathies

As the defects for several nuclear envelopathies present late in adolescence, one hypothesis for a disease mechanism is the failure of satellite/stem cells. Some emerin mutations that cause Emery-Dreifuss muscular dystrophy (EDMD) double the length of the cell cycle when expressed in cultured cells [152]. This finding alone could not explain the pathology of the disease because other EDMD-associated emerin mutations showed no increase in cell cycle duration. However, these other emerin mutations also likely yield some cell cycle defects as pRb pathways are misregulated in NE-related muscular dystrophies [153, 154].

6.2 Lamin A-LAP2α-pRb regulation of entrance into S-phase

The interaction between lamin A and pRb also involves LAP2α, a soluble splice variant of the gene that encodes the NET LAP2β [144], thus it can occur in the nucleoplasm as well as at the NE [155]. These interactions presumably sequester pRb complexes and stabilize them by protecting them from degradation by the proteasome [18]. pRb negatively regulates E2F-dependent transcription controlling the balance beween proliferation and differentiation. When cells are preparing to divide pRb becomes phosphorylated, first by cyclinD/cdk4 and cdk6 and later by cyclinE/cdk2. Phosphorylation of pRb releases the E2F transcription factor from the complex so it can activate S-phase progression genes [156, 157]. In contrast, hypophosphorylated pRb sequesters E2F early in the G1 phase of the cell cycle and

keeps cells from actively cycling (reviewed in [158]) (Fig. 3). Overexpression of LAP2α stabilizes the pRb/E2F complex so that E2F is never released, presumably by sequestering the total pool of pRb/E2F with the more abundant lamins, and resulting in cell cycle arrest [144, 159]. Knockdown of lamin A yields a similar outcome, possibly by destabilizing LAP2α/pRb/E2F complexes when they lose their anchor [160]. This would deplete the pool of E2F that could be released when cyclin/cdk kinases are activated. To parallel LAP2α overexpression resulting in cell cycle withdrawal, depletion of LAP2α in cultured fibroblasts stimulates cell proliferation [160]. Depletion of LAP2α in mice yields cell hyperproliferation, though intriguingly focused on erythroid and epidermal progenitors [161]. These tissues/cell types are already highly proliferative, indicating that the threshold levels of hyperphosphorylated free pRb needed to activate S-phase progression are more likely to be affected by lamin A/LAP2α levels in cell types already under strong regulation. Thus the lamin A/LAP2α/pRb nexus appears to fine-tune this regulation. These findings are further consistent with the hypothesis that reduction in lamin A levels would facilitate rapid cell proliferation and tumor development.

6.3 Integral NE protein regulation of cell cycle progression and withdrawal

A *C. elegans* member of the nesprin family of NETs, KDP-1, is important for cell cycle progression between the end of S phase and entry into mitosis [162]; however, the mechanism of how it affects cell cycle timing remains unknown.To investigate whether these reported links between the NE and the cell cycle were rare functions for the NE or could be viewed as the proverbial tip of the iceberg, a recent study ran 39 novel NETs through a FACS-based cell cycle screen. The aim was to identify those NETs whose exogenous expression in 293T/HEK cells could alter the 4N/2N DNA ratio, representing the G2/M and G1/S-phase populations respectively [151]. Most of the NETs had no effect. However, 8 of these NETs — one fifth of those tested — yielded significant effects with seven increasing and one decreasing the 4N:2N ratio [151]. Intriguingly, most of the NETs that altered the cell cycle profile showed a marked degree of tissue-specificity in their expression, being expressed preferentially in one or a few tissues only. Perhaps this was to be expected as these NETs had been identified in proteomic analyses of NEs isolated from particular tissues [31, 163], but it was nonetheless surprising that proteins involved in as central a function as the cell cycle would be tissue-specific.

Most of the NETs did not appear to interface with common pathways linked to cell cycle regulation as their phenotypes were unchanged in cells lacking pRb or p53

[151]. In contrast, the effect of NET59/Ncln was lost in $p53^{-/-}$cells. NET59/Ncln has been linked to TGFβ signaling pathways through an indirect interaction with Smad proteins [164]. NET4/Tmem53, which was among the most tissue-specific NETs, also lost its effect in cells deficient for pRb or p53. NET4/Tmem53 knockdown promoted cell cycle withdrawal not by affecting total pRb levels (as did lamin A), but by dramatically reducing the level of phosphorylated pRb [151]. Levels of p53 were doubled in these cells while p21 levels were increased 7-fold. These events all depend on the p38 MAP kinase, which somehow is activated after NET4/Tmem53 loss. This is particularly interesting because NET4/Tmem53 appears to be restricted to the outer nuclear membrane while NET59/Ncln is in the inner nuclear membrane [165]. Thus, NETs on both sides of the NE can influence the regulation of the cell cycle by different mechanisms. That three quarters of the NETs that affected the cell cycle were tissue-specific to a certain degree, and did so by novel or less characterized pathways that do not involve master regulators, suggests they might be important for tumors affecting the tissues where these NETs are expressed.

7. The nuclear envelope and mitosis

The NE is disassembled in the mitosis of all higher eukaryotes, thus allowing tubulin access to assemble the mitotic spindle. Failure to completely disassemble the NE could result in remaining connections increasing the incidence of lagging chromosomes and aneuploidy, a common feature of cancer cells. Mitotic kinases hyper-phosphorylate lamins to drive the disassembly of the lamin polymer [166-169]. Blocking this step also blocked the cell cycle [167]. At the time of these observations it was thought that this made NE proteins irrelevant for mitotic events after prophase: it was a full ten years before the question was asked “what happens to NE proteins during mitosis?”

Many NE proteins appear to have separate functions in mitosis. Ran, critical for cargo release and receptor shuttling in nucleocytoplasmic transport (reviewed in [5]), and the transport receptor importin/karyopherinβ are also necessary for aster formation in the mitotic spindle [170, 171]. Some NPC structural components appear to have mitotic functions as well, for instance the Nup107-160 complex associates with kinetochores during mitosis [172].. Lamins are also affected by the Ran-Importin nexus as dominant-negative lamin mutants interfered with spindle assembly [173]. This may indicate a supporting function of the lamin polymer in spindle formation.

Most NETs are distributed throughout the mitotic cell, but excluded from the chromosomes and spindle area [32]. Nonetheless, a small subset of NETs (Samp1/Tmem201/NET5, WFS1 and Tmem214) have been observed on the mitotic spindle [32, 174]. The function of this localization is not clear. One possibility is that it is a non-functional association of mitotic vesicles containing NETs that connect the NE to microtubules during interphase or that functionally interact when microtubules penetrate the NE in initiating NE reassembly at the end of mitosis as NET-chromatin interactions are thought to target mitotic vesicles onto chromatin to facilitate NE assembly [175, 176]. Defects in any of these mitotic functions could affect the quality of cell division and lead to aneuploidy, a common feature of tumors.

8. Revisiting the nuclear lamina as a prognostic cancer marker and the nucleo-cytoskeletal connection

Loss of differentiation in cancer cells, a process by which cells lose their tissue-specific characteristics and become more stem-cell like, has long been accepted as a hallmark of the disease [177]. The differentiation status of tumors has proven useful as a prognostic marker in certain cancers [178]. Loss of lamin A has been observed in many tumors. Though the reason and consequences of this loss are not certain, it may reflect a loss of differentiation during tumorigenesis, However, instead of representing “retrodifferentiation”, the absence of lamin A could indicate that the original cell that generated the tumor never expressed Lamin A in the first place because it was a stem cell. This makes sense for tumors where the absence of lamin A correlates with increased metastasis, but would seem inconsistent at first glance with the reports of tumor types where the expression of lamin A correlates with their metastatic potential. A recent study on colorectal carcinoma (CRC) has resolved this conundrum and suggests that lamins do indeed play an important role in tumorigenesis [179].

Stem cells reside at the base of the crypts in colonic epithelium, and express lamin A. Then, in the transit amplifying zone lamin A expression is lost, resembling the situation in early development where lamin A is expressed in oocytes but disappears after several cell divisions [37]. Finally, at the top of the crypt, both lamin A and lamin C are detected in the fully differentiated mucosa (Fig. 4). In CRC lamin A expression is associated with an increased mortality risk [179], apparently because the tumors arise from the lamin A-expressing stem cells at the base of the crypt

rather than the more differentiated lamin A-expressing cells at the top. In general, expression of lamin A slows cell proliferation while its absence promotes proliferation [18, 159], but no difference in the proliferation rate was found between model CRC lines expressing or lacking lamin A [179]. However, cells expressing lamin A were significantly more invasive, in agreement with the two-fold increased mortality risk observed in patients with lamin A positive tumors. This finding has made lamin A an important prognostic biomarker in CRC, but the study additionally revealed a mechanism by which lamin A expression might increase metastatic potential.

The enhanced invasive properties of lamin A-positive cells can be explained because of lamin A/NE effects on gene expression. Lamin A promotes upregulation of the T-plastin gene (PLS3) and downregulation of E-cadherin. Plastins (also called fimbrins) are actin-binding proteins that have already been described as metastatic promoters, affecting cytoskeleton organization and cell motility [180, 181]. Thus, having lamin A in these tumors weakens cell adhesion and enhances cell motility, consequently increasing the invasive potential.

In addition to lamin effects on gene expression, it is also possible that alterations in the connections between the peripheral nucleoskeleton and the cytoskeleton would affect cell motility and the degree of agressiveness of a tumor. The LINC complex binds to nuclear and cytoskeletal components. It is formed by the interactions of two NET families: the SUN-domain and the Syne/Nesprin proteins [86]. Disruption of this complex leads to defects in the mechanical stability of cells, tensional integrity defects in connectivity, and defects in cell mobility and mechanotransduction [182, 183]. Mechanotransduction signaling has emerged in the past decade as an important factor that can promote tumorigenesis and metastasis [184]. Since the nucleo-cytoskeletal connection is a driving force in mechanical transduction signaling, this suggests an important role for the NE in cancer development.

9. Concluding remarks

The diversity of NE functions in genome regulation, genome stability, cytoskeletal stability, cell migration, and cell cycle regulation support many ways that NE proteins can contribute to cancer development (summarized in Fig. 2). The recent finding of so many tissue-specific NETs [31, 32, 165] suggests that the NE could further contribute to the characteristics unique for different tumor types.

Some NETs are differentially expressed in cancer, relative to normal tissues. We investigated the gene expression profiles of lamin A, B1 and B2 and also of 29 NETs that had been verified by our lab and others [31, 32, 163, 165, 185-194] in a database of tumor and normal samples available at BioGPS [73, 74]. Whilst most of the NETs have variable levels between samples, several were generally up- or downregulated in all cancer samples relative to the normal tissues. Lamins B1 and B2 as well as nucleoporin gp210 were generally upregulated, possibly reflecting a greater need for them in faster cycling cancer cells. In contrast, both nesprin 1 and 2 and METTL7A were downregulated in the nine tumor types tested (Fig. 5A). Another NET often affected is SCCPDH/NET11, which was previously linked to cell cycle regulation [151].

As many NETs are preferentially expressed in certain tissues [31, 32, 165], they have the potential to be used as markers for certain tumor types. For example, a comparison of the profiles of lung and ovary cancers (Fig. 5B) shows a very similar pattern overall. However, the NET LPCAT3 is generally downregulated in lung tumors, but clearly upregulated in all the ovary tumors. In normal ovary tissues LPCAT3 is not expressed, but it is in many other tissues including lung. The NET TM7SF2/NET47 is a sterol reductase expressed preferentially in heart, brain and liver tissues [72, 73], and it also plays a role in spatial chromosome organization (Zuleger N. & Schirmer E., unpublished results). Interestingly, NET47 appears to be largely unaffected in cancers from most tissues, but it is downregulated in most liver cancer samples tested [73, 74]. Finally, DHRSF7/NET50 is normally expressed in a small number of tissues including pancreatic islets, however its expression falls in pancreatic cancer samples [73, 74]. Whilst these are only a few observations based on a relatively small study, it seems clear that a number of NE proteins, which may be involved in a wide range of functions as noted throughout this review, are differentially expressed in tumor cells. NETs may represent an as yet untapped reservoir of diagnostic and prognostic markers in cancer as well as having the potential to be future therapeutic targets.

Conflict of Interest Statement

The authors declare that there are no conflicts of interest

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Figure Legends

Fig. 1.

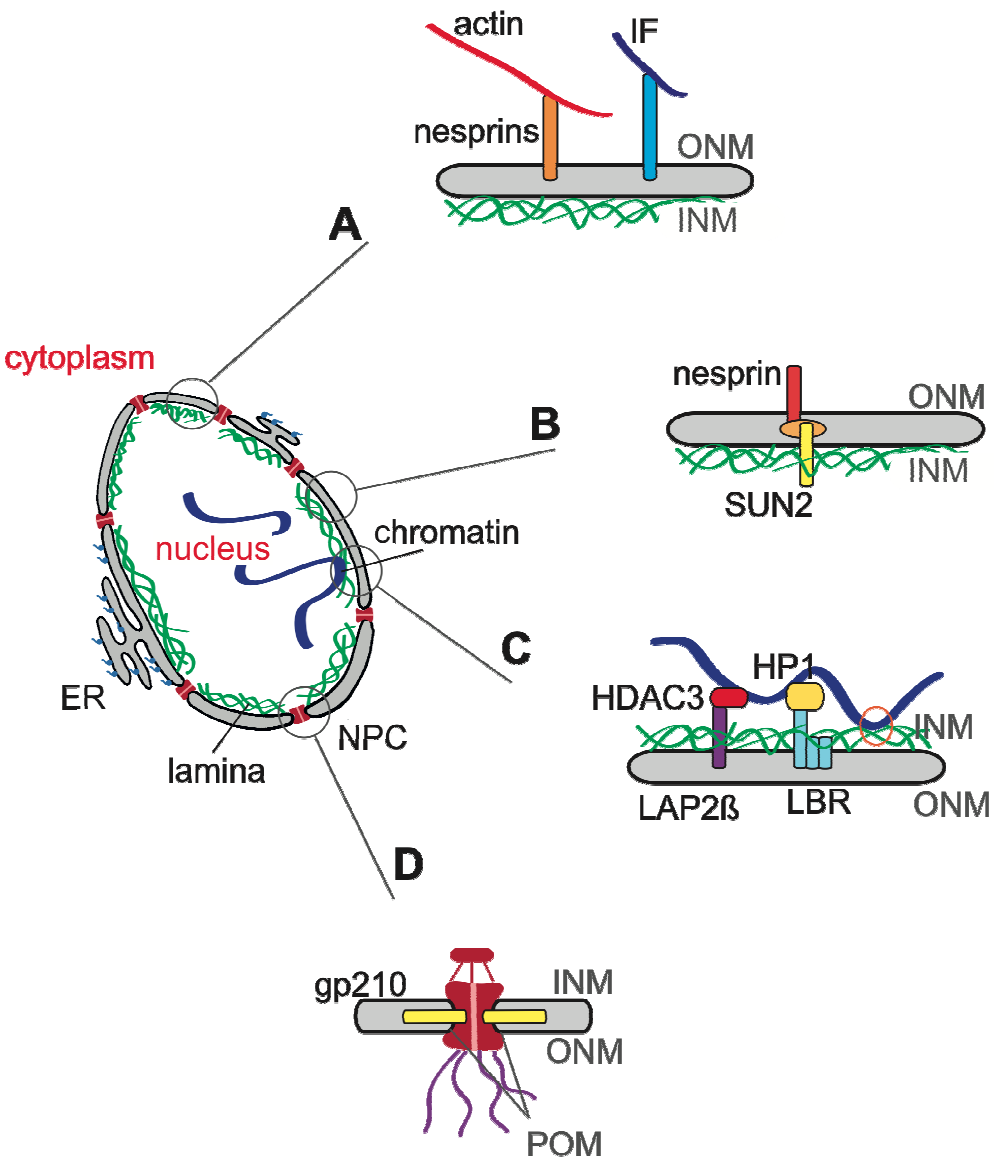
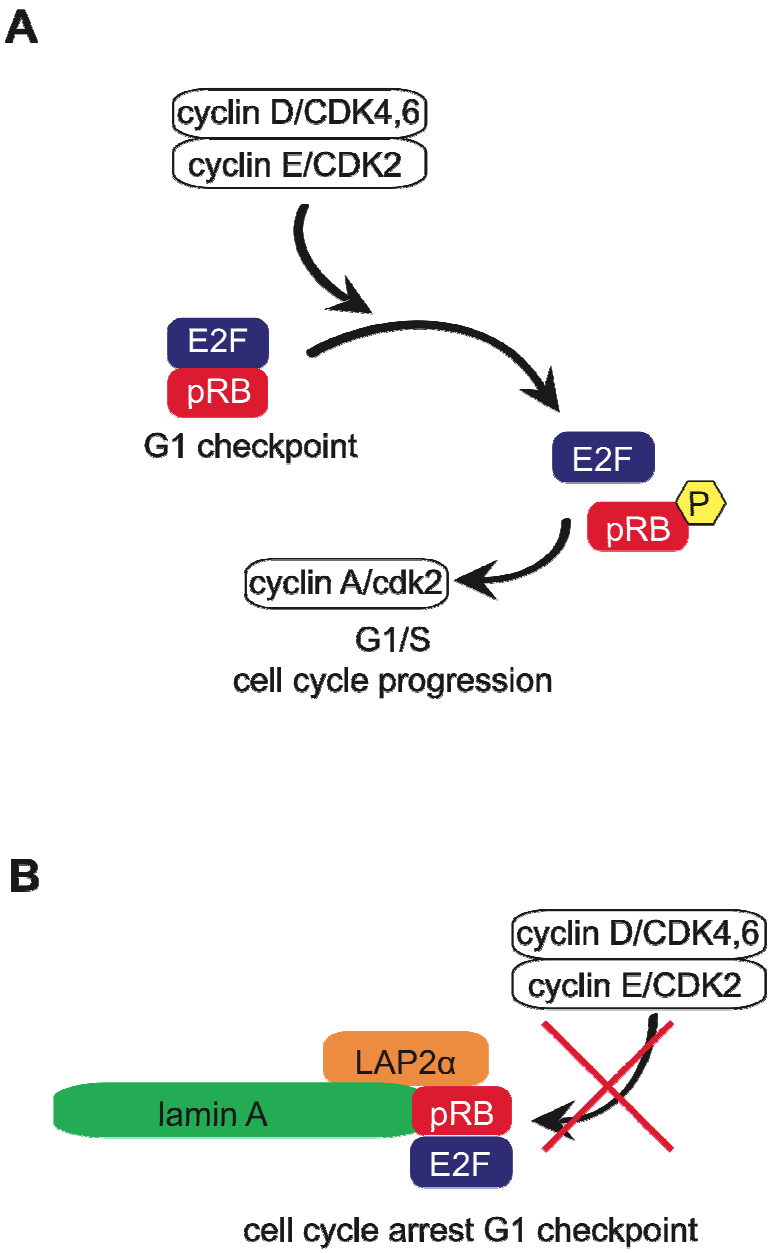


Diagram of the nuclear envelope. The nuclear envelope (NE) is a double membrane system that includes the nuclear lamina and a large number of transmembrane proteins (NETs). The inner nuclear membrane (INM) and the outer nuclear membrane (ONM) are joined at the nuclear pores by a pore membrane (PoM). The

ONM is continuous with the endoplasmic reticulum and is studded with ribosomes. A. The NE is connected to the cytoskeleton by nesprins inserted in the ONM. These interact with actin and intermediate filaments. B. The INM and ONM are also connected by the LINC complex, involving nesprins on the ONM and SUN-domain proteins on the INM. C. Chromatin is linked to the INM by interactions with NETs, such as LAP2β and LBR, which interact with HDAC3 and HP1 respectively. In addition, lamin A can bind core histones H2A/B and also DNA directly, although with much lower affinity. D. The nuclear pore complex (NPC) regulates transport in and out of the nucleus. The NET gp210 sits in the PoM and plays an important role in this process.

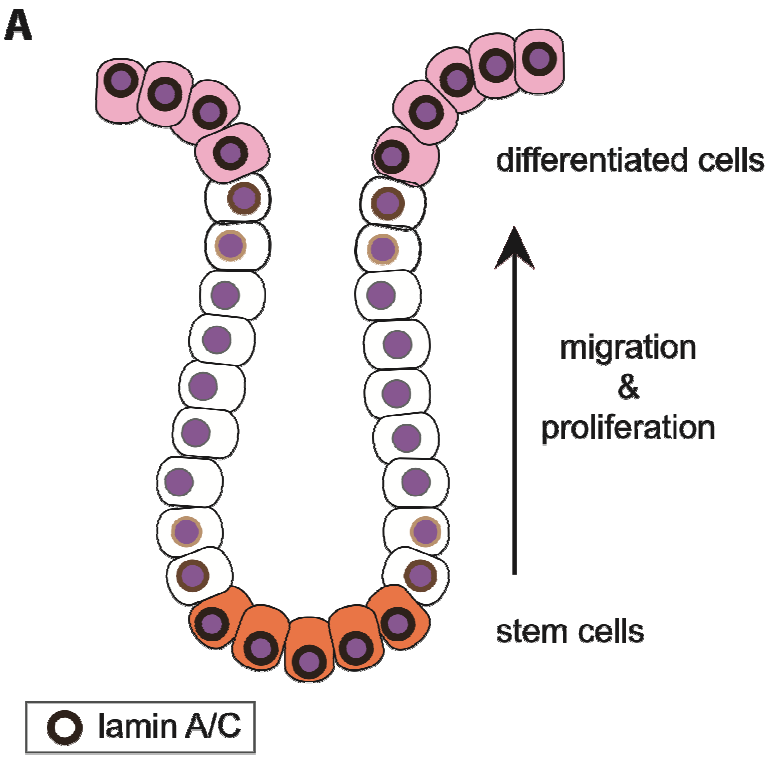
Fig. 2.



Summary of cancer cell defects and their nuclear envelope links. Tumor cells commonly show various aberrations, at a genetic and structural level. Nuclear architecture is often affected, with changes in nuclear size, volume and an increased likelihood to suffer ruptures. Genome stability is also affected, involving changes in chromatin organization. Cancer cells are often aneuploid and/or contain chromosome translocations. Finally, a variety of cellular processes such as DNA repair, mitosis,

regulation of cell cycle and signalling are often impaired. Proteins in the nuclear envelope, lamins and NETs, are involved in each of these functions.

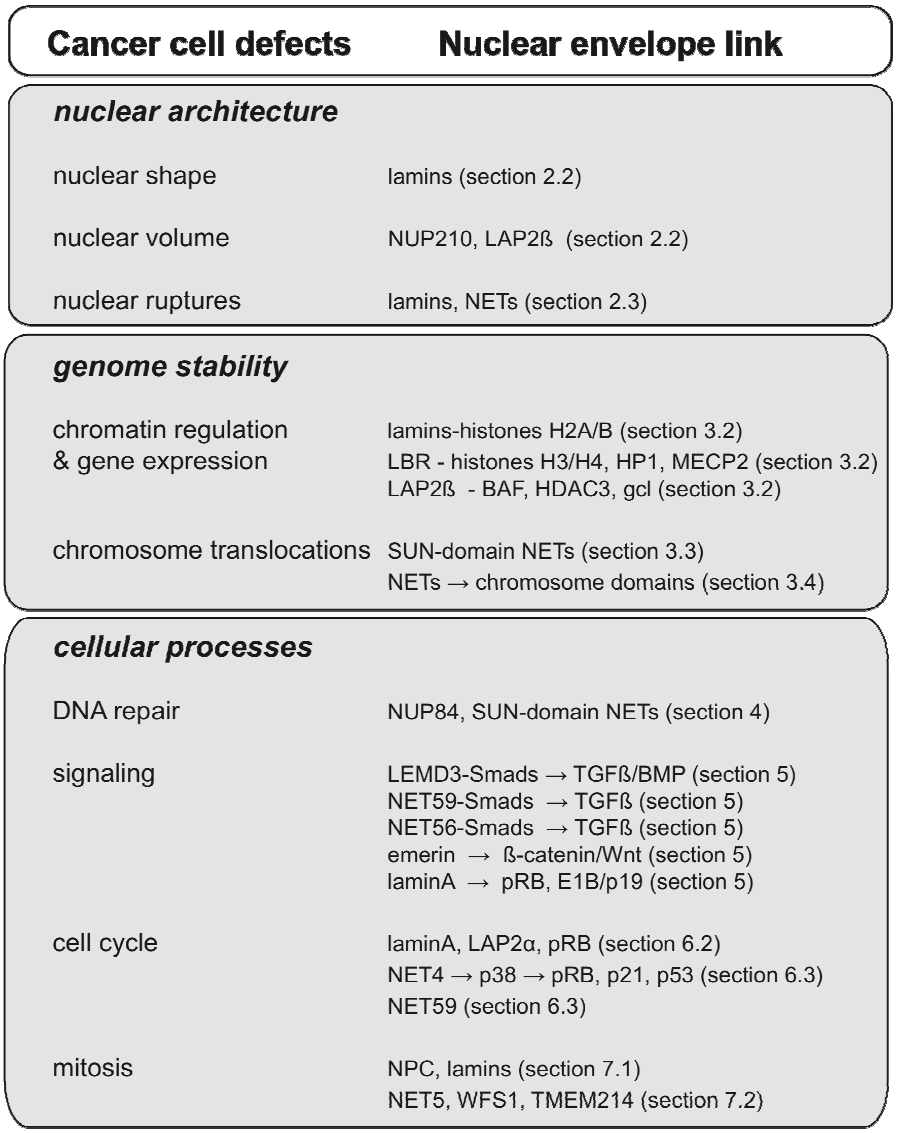
Fig. 3.



Regulation of the cell cycle is affected by NE proteins. A. The hypophosphorylated form of the retinoblastoma protein (pRB) binds the transcriptional activator E2F, preventing its function. The pRB-mediated G1 checkpoint is not passed until pRb is phosphorylated through the combined actions of cyclin D/CDK4,6 and cyclin E/CDK2. This allows E2F to dissociate from pRb and activate other factors, like cyclin A and proliferating cell nuclear antigen (PCNA), with consequent progression of cell cycle into S phase. B. Interaction of pRb with lamin A and LAP2α sequesters

the pRb/E2F complex, reducing the pool of available E2F and arresting the cell cycle at the G1 pRb-dependent checkpoint.

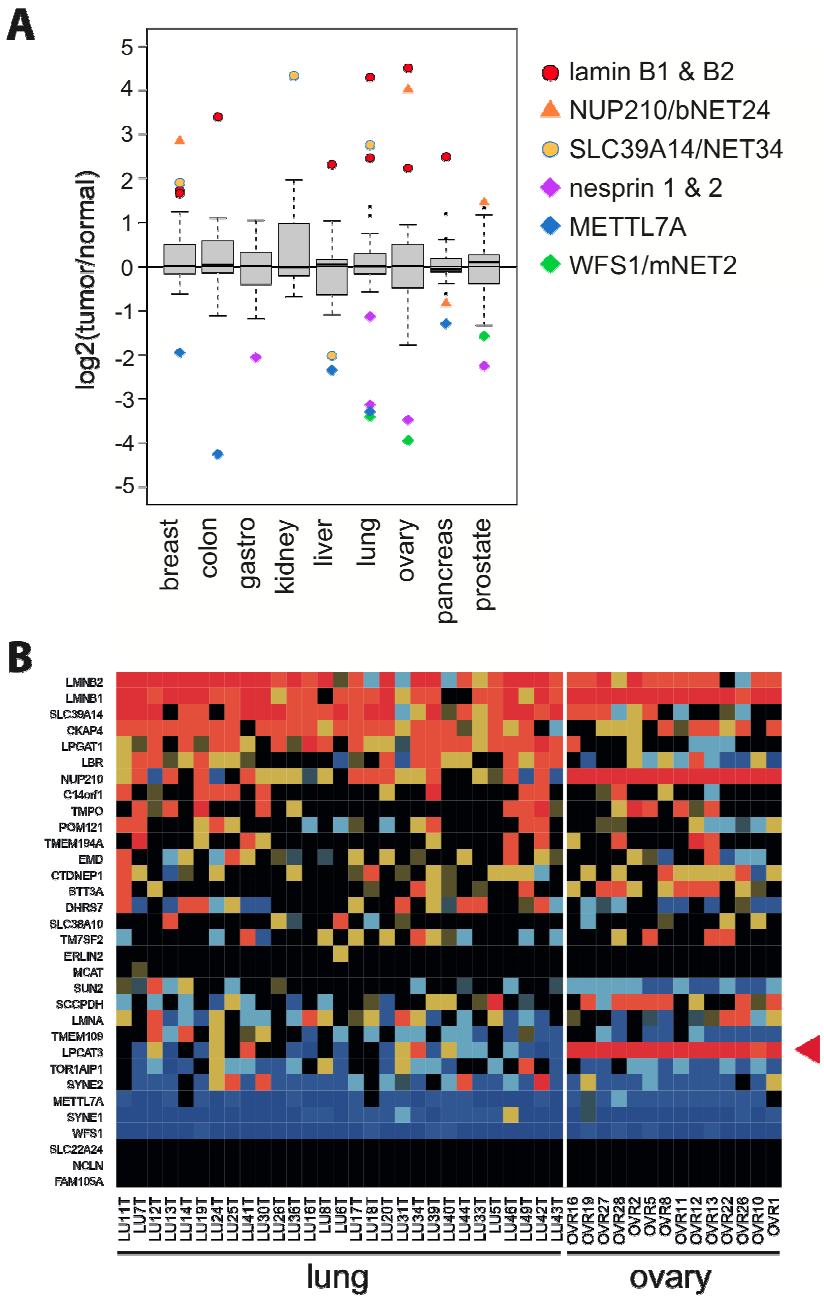
Fig. 4.



Lamin A/C expression is a prognostic biomarker in colorectal cancer (CRC). Expression of lamin A/C is strongly correlated with colorectal cancer mortality. A. Colonic stem cells are located at the base of the crypts, and express lamin A. As cells proliferate and migrate upwards lamin A expression disappears, to reappear again in the fully differentiated epithelium at the colonic mucosa. B. Proliferating cells in the crypt may give rise to tumors. Those expressing lamin A are associated with a

higher mortality rate, perhaps because lamin A positive cancer cells have a more stem cell-like phenotype, and consequently are more dangerous.

Fig. 5.



Tumors show aberrant expression of nuclear envelope proteins. A. Boxplot showing the distribution of log2(tumor/normal) signals for 29 NE proteins in nine tissues. The proteins that change the most, both up and downregulated, are generally the same ones, with lamin B1/B2 and gp210/NUP210 usually upregulated in tumors, while nesprins and METTL7A are almost always downregulated. However, some NETs such as WFS1, are strongly downregulated in some tumors but not others. Similarly, NET34 is strongly upregulated in breast, kidney and lung cancers, but strongly

downregulated in liver cancer. B. Heatmap showing the expression of 29 NE proteins in individual lung and ovary cancer patients in comparison to their normal counterparts. A palette of reds and blues indicate relative levels of up and downregulation, respectively. Most NE proteins tested vary between patients, however the most highly up and downregulated are shared between most patients and tumor types. Interestingly, the tissue-specific NET LPCAT3 (red arrowhead), which is expressed in most tissues but absent normal ovary, became strongly upregulated in all ovary cancer patients whilst generally being downregulated in lung cancer patients.