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## Role of the nuclear envelope on genome organization and gene expression

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While often depicted as a static structure upon which proteinaceous factors bind to control gene expression, the genome is actually highly mobile and capable of exploring the complex domain architecture of the nucleus, which in turn controls genome maintenance and gene expression. Numerous genes relocate from the nuclear periphery to the nuclear interior upon activation and are hypothesized to interact with pre-assembled sites of transcription. In contrast to the nuclear interior, the nuclear periphery is widely regarded as transcriptionally silent. This is reflected by the preferential association of heterochromatin with the nuclear envelope. However, some activated genes are recruited to the nuclear periphery through interactions with nuclear pore complexes (NPCs) and NPC components are capable of preventing the spread of silent chromatin into adjacent regions of active chromatin, leading to the speculation that NPCs may facilitate the transition of chromatin between transcriptional states. Thus, the nuclear envelope (NE) might better be considered as a discontinuous platform that promotes both gene activation and repression. As such, it is perhaps not surprising that many disease states are frequently associated with alterations in the NE. Here we review the effects of the nuclear envelope and its constituents on chromatin organization and gene expression.

While genome sequencing continues to enumerate the genomic potential of a multitude of organisms, understanding what genes are expressed, when they are expressed and how they are expressed are fundamental challenges for biology. But because, at any given time, all genes in a genome do not have the same potential to be expressed, predicting gene expression is not simply a matter of knowing what transcription factors are present and where they bind. As early as 1928, Emil Heitz noted at least two types of chromatin: highly condensed chromatin that failed to decondense during interphase and other regions on the same chromosome that did.<sup>1, 2</sup> He referred to these distinct chromosomal domains as heterochromatin and euchromatin, respectively. Eighty years later we know that DNA must be highly organized and packed for it to even fit in the nucleus of a cell and that tightly packed regions of chromatin are generally transcriptionally silent, whereas loosely packed chromatin has greater potential to be transcriptionally active. DNA organization of this sort is accomplished through multiple layers of compaction to form chromatin. The first order of

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compaction consists of 146 base pairs of DNA wound around an octamer of histone proteins to form a nucleosome. Nucleosomes oligomerize to form a 30nm fiber and ultimately organize into a poorly defined higher order chromatin structure. Each layer of compaction is highly regulated and subtle changes induced by chromatin binding proteins and histone modifying enzymes can greatly influence the accessibility of DNA to transcription factors, leading to regions that are transcriptionally active or repressed, as is the case for Emil Heitz's euchromatin and heterochromatin, respectively (reviewed in<sup>3, 4</sup>). The N-termini of histones are extensively post-translationally modified by acetylation, methylation, phosphorylation, ubiquitination or sumoylation. Hyperacetylation of lysine residues is correlated with increased transcription, particularly acetylation of lysine 16 on histone H4 (H4 K16<sup>ac</sup>) whereas lysine hypoacetylation and methylation of lysine 9 on histone H3 (H3 K9<sup>me</sup>) are hallmarks of heterochromatin (reviewed in<sup>5, 6</sup>). To prevent the spread of one chromatin state into an adjacent region of the opposite chromatin state, boundary regions are present that lie at the interfaces of opposing chromatin. Such boundary regions are often dynamic and contain epigenetic marks of both active and inactive chromatin.<sup>7-9</sup>

Early electron micrographs revealed that although these highly condensed chromatin domains are found both at the nuclear periphery and nuclear interior, for many cell types they are preferentially localized to the nuclear periphery, suggesting, that regions interior to the nucleus tend to promote gene expression, while regions at the periphery tend to promote gene silencing. An exception to this generality (which has been evident since these early images) is near nuclear pore complexes – complex macromolecular structures embedded in the nuclear envelope that permit and regulate macromolecular transport between the nucleus and the cytoplasm. While these morphological characteristics have been a source of speculation regarding gene expression<sup>10, 11</sup>, the molecular mechanisms and functional impact of the three-dimensional nuclear architecture on gene expression is only now beginning to be understood.

The nuclear envelope (NE) is a double-membrane system surrounding the nuclear chromatin. It is comprised of an outer nuclear membrane, which is continuous with the endoplasmic reticulum, and an inner nuclear membrane exposed to the nucleoplasm. Underlying the inner nuclear membrane in metazoan cells is the nuclear lamina, a complex meshwork of intermediate filaments comprised of lamin A, C, B1 and B2, which connect to the NE through multiple integral inner membrane proteins (reviewed in<sup>12-15</sup>). The primary function of the lamina has been thought to maintain nuclear shape in concert with the NE, however in recent years it has become clear that the lamina has important functions in transcriptional regulation and genome organization.<sup>12, 16, 17</sup> Mutations that affect the functions of lamins often lead to a diverse set of tissue specific diseases referred to as laminopathies, which include forms of muscular dystrophy, lipodystrophy and premature ageing syndromes (reviewed in<sup>18-22</sup>).

Approximately 30 different nuclear pore proteins (nucleoporins or nups) comprise each of the numerous, large, and proteinaceous nuclear pore complexes (NPCs) that are distributed throughout the nuclear envelope, thereby providing gateways for exchange between the nucleoplasm and cytoplasm. Each NPC is highly modular and symmetric in structure, forming an octagonal donut-like structure embedded in, and spanning the double membrane of the NE. Consistent with the morphological symmetry, each nucleoporin is present in multiple (at least 8) copies.<sup>23</sup> While the majority of nucleoporins are symmetrically located on both the cytoplasmic and nucleoplasmic sides of the NPC, there are those that are localized specifically to either the cytoplasmic or nucleoplasmic face of the NPC. On the cytoplasmic side, these nucleoporins (e.g. Nup159p and Nup82p in yeast or Nup214 and Nup88 in mammals) form flexible filaments that emanate into the cytoplasm apparently like tentacles of an octopus. On the nucleoplasmic side, (Nup1p, Nup2p and Nup60p in yeast or

Nup50, and Nup153 in mammals) and associated proteins (Mlp1p and Mlp2p in yeast or Tpr in mammals) form a basket-like structure that can extend upwards of ~100 nm into the nucleus and is situated in a prime location to facilitate interactions with underlying chromatin (Figure 1).<sup>24, 25</sup>

Consistent with early morphological observations, localization of specific chromosomal regions within the nucleus revealed that transcriptionally inactive regions such as telomeres, centromeres, the inactive X chromosome and in yeast, the stably repressed mating type loci (*HMR* and *HML*) are preferentially associated with the nuclear periphery. Moreover, genes activated during cellular differentiation often translocate away from the periphery towards the nuclear interior, as is the case for immunoglobulin loci during B-cell activation.<sup>26, 27</sup> However, the nuclear periphery can also promote transcription as demonstrated by numerous yeast genes that are recruited to the nuclear periphery for full transcriptional activity.<sup>28-34</sup> The mechanism(s) that determine whether a peripherally localized gene is active or repressed are not clear, but likely depend on the context of the surrounding nuclear architecture including interactions with specific subdomains of the NE, contacts with the nuclear lamina in metazoans and the state of the surrounding chromatin.

How are these epigenetic states (and expression) of the chromatin influenced by the three dimensional architecture of the nucleus? Considering the multitude of parameters influencing the epigenetic states, it seems impractical to think we can generalize what is learned from single genes to the rest of the genome. One promising approach that is beginning to reveal principles governing gene expression involves systems biology (Box 1). In such an approach, we can consider the influence of directed perturbations on a genome-wide scale. We can also begin to consider an integrated approach involving global interrogations of the dynamics of protein-DNA interactions, molecular epigenetic marks on chromosomes, expression states etc., and to explore these data computationally to build models of function that can begin to explain how the structural dynamics of the genome and the cell influence and control expression of the cell's blueprint.

## Nuclear envelope associated heterochromatin

### Role of the nuclear envelope in metazoan differentiation

Advances in microscopy techniques have revealed the dynamic nature of the chromosomal domains. *In-vivo* visualization and real-time tracking of discrete chromosomal loci (Figure 2) in mammalian cells have led to the discovery that chromosomes encoding relatively few genes, said to be gene-poor, are more frequently associated with the nuclear periphery whereas gene-rich chromosomes are positioned more internally.<sup>35, 36</sup> Similarly, highly transcribed, co-regulated genes often cluster within chromosomal territories, as is the case for the murine immunoglobulin gene clusters *Igh* and *Igk*, encoding genes of the immunoglobulin heavy and light chains respectively, which, in hematopoietic progenitor B-cells, associate with the nuclear lamina when inactive and relocate away from the nuclear periphery following commitment to the B-cell lineage.<sup>26, 27</sup> In this context, peripheral localization is proposed to prevent spurious immunoglobulin rearrangements by limiting accessibility of *Ig* loci to recombination factors.<sup>37</sup> Similar gene repositioning occurs with the *MASH1* locus during neural induction of embryonic stem cells and the *beta-globin* genes during erythrocyte maturation.<sup>38, 39</sup> *NANOG* also undergoes gene repositioning during cell differentiation; however, in this case, because *NANOG* expression is required to maintain pluripotency of human embryonic stem cells, upon differentiation *NANOG* becomes transcriptionally silent and relocates from the nuclear interior to a more peripheral position.<sup>40</sup> These observations correlate changes in gene positioning with changes in transcriptional activity during cell differentiation, and raise the question of whether gene relocalization is the cause or consequence of gene activation.

### Silent domains at the nuclear periphery: lessons from yeast

Although budding yeast lack the hallmarks of heterochromatin found in higher eukaryotes such as H3 K9<sup>me</sup> and the compaction required to visualize heterochromatin by microscopy, silencing nonetheless occurs in heterochromatin-like regions and this model organism has been instrumental to our understanding of the importance of gene positioning in transcriptional regulation. Silent regions in yeast including centromeres, telomeres and the silent mating type loci (*HMR* and *HML*), all localize in the vicinity of the nuclear periphery.<sup>41-43</sup> Yeast telomeres are assembled into silent chromatin and coalesce to form clusters of 8-10 foci per cell.<sup>44-46</sup> These telomeric foci are tethered at the periphery through two redundant mechanisms involving interactions between Sir4p, a component of the silencing machinery, and the peripheral inner nuclear membrane protein, Esc1p or between the DNA-end binding yKu heterodimer and the integral inner nuclear membrane protein Mps3p (reviewed in<sup>47-49</sup>). Combinatory deletions that negate both pathways lead to dispersal of telomere foci into the nuclear interior.<sup>50</sup> Microarray analysis revealed a loss of subtelomeric silencing concomitant with decreased expression of non-telomeric genes, a result attributed to dispersal to ectopic chromatin sites of the silent information regulatory complex (Sir2p-Sir3p-Sir4p) that is required for silencing.<sup>50</sup> Therefore, it was concluded that telomere clustering at the periphery sequesters a limited pool of Sir proteins at concentrations high enough to permit heterochromatin formation. In support of this, silencing of an *HMR* locus flanked by weakened silencing elements is enhanced when artificially tethered to the nuclear periphery and enhanced silencing is dependent on intact peripheral telomere foci.<sup>51</sup> However, peripheral tethering of an *HMR* locus lacking silencing elements failed to repress, suggesting that a peripheral localization promotes but is not sufficient for repression.

### Nuclear lamina is linked to chromatin and gene expression

In higher eukaryotes, a mesh-like network lies directly beneath the nuclear envelope. This nuclear lamina structure is comprised of intermediate filament proteins called lamins, which are anchored to the inner nuclear membrane through associations with integral membrane proteins or through membrane insertion of a lipid moiety. The nuclear lamina is positioned to be a key regulator of gene expression through physical organization of the genome or by acting as a scaffold for dynamic interplay of effectors with the genome. Accordingly, *in vitro* binding studies suggest that lamins physically interact with the genome either through binding of histones or by directly binding to specific DNA sequences termed matrix attachment regions (MARs).<sup>52, 53</sup> The first *in vivo* high resolution mapping of interactions between the genome and the nuclear lamina were performed in the fly *Drosophila melanogaster* using a genome-wide approach termed DamID (Box 2).<sup>54</sup> In brief, the *Escherichia coli* adenine methyltransferase was fused to the B-type lamin (Lam) to generate a lamin chimera with DNA methylation activity. The chimera was expressed *in vivo* and DNA that came in physical proximity to the B-type lamin was methylated on adenine residues. Subsequent specific isolation of methylated adenine DNA fragments followed by amplification, labeling and hybridization to microarrays resulted in the identification of 474 genes organized into 52 gene clusters that are targets of Lam binding. Microscopy analysis to determine nuclear gene positioning confirmed that Lam target genes were more likely to have a peripheral localization than non-Lam target genes. Importantly, genome wide transcriptional profiling suggested that Lam target genes are expressed at lower levels than non-target genes and chromatin immunoprecipitation revealed that lamin-associated chromatin is reduced in the histone modifications H3 K4<sup>me3</sup> and H4 K16<sup>ac</sup>, which are associated with active genes.<sup>54</sup> Furthermore, upon induction of differentiation the expression level of genes newly associated with Lam decreased, and expression of genes that lost Lam association increased, consistent with the hypothesis that transcriptionally inactive genes associate with lamins at the nuclear periphery. Surprisingly, further ChIP experiments showed that (at least in flies) the Lam-associated transcriptionally inactive genes adopt a

chromatin state distinct from 'classical' heterochromatin. Specifically, heterochromatin markers, heterochromatin protein 1 (HP1) and Su(var)3-9 (recently renamed, Drosophila lysine methyltransferase 1 (dKMT1)) were not found at Lam target genes.

What are the determinants of Lam binding? The Fornerod laboratory correlated Lam binding with regions of late replication, low transcriptional levels and an absence of active histone marks.<sup>54</sup> A histone deacetylase inhibitor reduced global Lam association, consistent with the finding that Lam binding sites are often flanked by long, intergenic hypoacetylated regions, suggesting that hypoacetylation promotes interaction with the lamina. However, the histone deacetylase HDAC3 also binds to the nuclear periphery, through the lamin associated protein Lap2 $\beta$  in mammalian cells.<sup>55</sup> Thus, raising a familiar question: Are the chromatin characteristics of Lam binding sites the cause or consequence of Lam association? Peripheral HDAC3 activity could be required to form hypoacetylated chromatin thereby promoting Lam binding, or alternatively, hypoacetylation and gene inactivation may occur as a consequence of chromatin recruitment to the nuclear periphery through association with Lam. In the latter case, Lam-association may be stabilized by hypoacetylation, establishing local feedback.

## Nuclear pore complexes: gene activation and chromatin boundaries

### Activated genes associate with nuclear pore complexes

Over the last decade it has become increasingly evident that highly transcribed genes associate with nuclear pore complex proteins in yeast. Genome-wide chromosome immunoprecipitation studies (ChIP-chip) have been performed on several components of the nuclear pore complex as well as multiple karyopherins, - the soluble proteins that mediate nuclear transport.<sup>56</sup> These studies revealed that the NPC components Nup2p, Nup60p, Nup166p, Nic96p, Mlp1p and Mlp2p and the karyopherins Cse1p, Kap95p and Xpo1p preferentially associate with actively transcribed genes, enriching with genes that are highly transcribed such as those involved in glycolysis and ribosome biogenesis. In contrast the Ran guanine-nucleotide exchange factor (RanGEF), Prp20p, which is mechanistically and physically linked to the dynamics of the nuclear import and export cycle, preferentially associates with inactive genes.<sup>56</sup> Prp20p is an essential transport factor that promotes the dissociation of karyopherin-cargo import complexes in the nucleus and associates with chromatin.<sup>57-59</sup> Prp20p association with inactive genes has been proposed to target newly imported transcription factors directly to target gene promoters.<sup>56</sup> Under repressive conditions the galactose-inducible *GAL* genes associate with Prp20p, but not with nucleoporins. Following the addition of galactose, the *GAL* genes associate with nucleoporins, but no longer associate with Prp20p, suggesting that a subset of genes can be recruited to the NPC under conditions of transcriptional activation.<sup>56, 60</sup>

In a different approach to identify genomic binding sites of nucleoporins, a chimera was generated between Nup2p and micrococcal nuclease (MN) whereby the nuclease activity of the chimeric protein can be activated by the addition of calcium ions (Box 3).<sup>61</sup> Upon expression of the chimera in yeast and subsequent activation of the nuclease by calcium addition, the chromatin in the vicinity of the Nup2-MN chimera (and presumably in the vicinity of Nup2p under normal conditions) is cleaved. Mapping of the double-stranded breaks allowed identification of Nup2p binding sites within promoter-proximal regions, referred to as Nup-promoter interactions (Nup-PIs). In the case of the *GAL* genes, Nup-PI appears to be an early event in gene activation as association with the promoter region required induction by galactose and the transcriptional activator Gal4p, yet occurred independently of recruitment of the SAGA complex or RNAPII. These observations point to a complex network of dynamic interactions that exist between actively transcribed genes and nucleoporins, presumably reflecting the association of these genes with the nuclear pore

complex. It should be stressed however, that although many active genes have been identified in association with NPCs, interaction with the nuclear periphery is not a mandatory feature of gene activity in yeast.<sup>62</sup> Some genes appear targeted to the periphery independent of transcription<sup>63</sup>, not all highly expressed genes associate with the periphery and it seems unlikely that genes would associate *stably* with the nuclear pore complex itself during expression. There are certainly dynamics, gene specific parameters and accompanying chromatin and transcriptional regulators involved and the mechanisms are yet to be revealed.

Nevertheless, chromatin association with the NPC is a feature of higher organisms as well as yeast. ChIP studies of Nup93 revealed association with transcriptionally inactive regions and chromatin domains bearing histone modifications indicative of heterochromatin.<sup>64</sup> Inhibition of histone deacetylases (HDACs) promotes global changes in transcription and the promoters of genes that increase in expression, also become associated with Nup93. In *Drosophila*, the hyperactivation of the X-chromosome in males is associated with peripheral localization and NPC association<sup>65, 66</sup> and the HSP70 locus associates with the NPC and localizes at the nuclear periphery.<sup>67</sup> Most recently, two papers have reported nucleoporin contacts with both silent loci and developmentally regulated genes undergoing transcriptional induction in *Drosophila melanogaster*.<sup>68, 69</sup> However, like yeast Nup2p, some *Drosophila* nups are highly dynamic and exchange between NPCs and the nucleoplasm, and these studies demonstrated that soluble intranuclear pools of some nups can interact with chromatin. Thus nup-chromatin interactions are not necessarily restricted to the nuclear periphery. These observations provide an important advance in our understanding of the basic roles of nucleoporins in gene regulation of higher eukaryotes and suggest that nucleoplasmic nups could play an active role in gene regulation, perhaps as direct regulators, or as scaffolds to bring modifiers, transcription factors and the genome in proximity. Nonetheless, given the complexities of gene regulatory mechanisms governing development, it is reasonable to assume that remodeling of NPC-chromatin contacts could represent yet another layer of complexity on the modulation of gene expression.

In yeast, visualization of inducible genes (*INO1*, *HXK1*, *GAL1*, *GAL2*, *SUC2*, *FIG2* and *HSP104*) through chromatin tagging confirmed that these loci relocate to the nuclear periphery and nuclear pore complexes upon transcriptional activation.<sup>28-30, 32, 56</sup> However, the molecular mechanisms and functional significance of recruitment are not well understood. What factors mediate gene recruitment to NPCs? Several laboratories have sought to address this question and have uncovered a diverse set of factors that contribute to chromatin-NPC interactions including transcription factors, chromatin modifying complexes, a histone variant, and mRNA processing and export factors.<sup>28, 29, 32, 61, 65, 70-73</sup> With respect to the NPC, it seems the nuclear basket proteins are important for gene recruitment. Nup2p binds the nuclear basket through Nup60p, interacts with chromatin through association with chromatin-bound Prp20p and is required for peripheral gene recruitment of *GAL1* and *INO1*.<sup>57, 63, 74, 75</sup> Also anchored to the nuclear basket via Nup60p are the myosin like proteins Mlp1p and Mlp2p that form coiled-coils that extend beyond the NPC and into the nuclear interior.<sup>24, 76</sup> Mlp1p is involved in gene targeting as deletion of *MLP1* prevents recruitment of *GAL10* and *HSP104* to the periphery.<sup>32</sup> Affinity purification of Mlp1p coupled with mass spectrometry revealed a physical interaction with the Spt-Ada-Gcn5 histone acetyltransferase complex, SAGA.<sup>77</sup> Moreover, ChIP experiments revealed that Mlp1p and SAGA components associate at the same promoter region of the *GAL* genes and that Mlp1p association is dependent on the integrity of the SAGA complex.<sup>77</sup> Interestingly, the SAGA component Sus1p is also present in a Sac3p containing mRNA export complex located at the nuclear pore complex and both Sac3p and Sus1p are required for efficient gene recruitment, providing further evidence that an NPC association is an important feature in peripheral gene targeting.<sup>29, 73</sup>

It appears that multiple aspects of transcriptional activation are required for NPC-chromatin interactions, however NPC-chromatin interactions are not required for all activation events. Therefore, a mechanism must exist that specifies which genes will adopt an NPC association. Such a mechanism is likely to be encoded by *cis*-acting DNA elements as peripheral targeting of some yeast genes are independent of transcription.<sup>61, 63</sup> A recent study identified two gene recruitment sequences (GRS I and GRS II) within the promoter region of the *INO1* gene responsible for *INO1* targeting to the NPC and were sufficient to target an ectopic locus to the nuclear periphery.<sup>78</sup> GRS I elements were identified in 94 promoters including several that are recruited to NPCs. The factors that bind GRS elements and promote recruitment remain a mystery but it remains only a matter of time before they too are identified to further inform the spatial organization and regulation of the genome.

### Nuclear pore complexes and transcriptional memory

In yeast a number of inducible genes are recruited to nuclear pores following induction. Interestingly, they appear to be retained at the periphery over multiple generations even after transcriptional repression.<sup>63</sup> Retention at the nuclear periphery during periods of repression is thought to serve as a memory of previous transcriptional activity to promote an accelerated transcriptional response following reactivation.<sup>79</sup> The fact that memory is maintained through multiple cell cycles indicates that the molecular mechanisms responsible are stable through DNA replication and mitosis and ultimately inherited by daughter cells. How do cells remember a previous transcriptional state? Epigenetic changes in chromatin structure are likely responsible. Alterations in nucleosome positioning mediated by the chromatin remodeling complex SWI/SNF and incorporation of the histone variant H2A.Z into assembled nucleosomes both contribute to transcriptional memory.<sup>63, 80</sup> An additional mechanism was identified by two recent studies that revealed a link between gene looping and transcriptional memory.<sup>81, 82</sup> Gene loops are dynamic structures formed upon transcriptional initiation through interactions between the promoter and 3'-end of a gene. Gene looping has been observed for both yeast and mammalian genes, however not all genes form loops.<sup>81, 83, 84</sup> Transcription-dependent gene loop structures were identified in two genes (*HXK1* and *GAL1*) that associate with NPCs during transcriptional activation and gene loops were maintained during intervening periods of transcriptional repression.<sup>81</sup> These DNA loop structures are termed memory gene loops (MGLs) and are required for transcriptional memory; mutations that prevent memory gene loop formation permit initial *GAL* gene activation, but transcriptional memory is lost. The molecular mechanisms that facilitate inheritance of MGLs to daughter cells during mitosis remain unclear. Strategies that establish transcriptional memory are likely to involve changes in chromatin structure through post-translational histone modifications and/or incorporation of histone variants that establish epigenetic marks for MGLs to reform in daughter cells upon completion of mitosis. How histone modifications can establish gene loops and maintain them to ensure their propagation through replication, mitosis and subsequent generations to act as memory markers remain key questions.

A role for the NPC in transcriptional memory is implicated by the association of memory gene loops with the nuclear basket protein Mlp1p.<sup>81</sup> Mlp1p associates with memory gene loops through interactions with both the 5' UAS and 3'-end regions, but not with internal regions of the *HXK1* gene. Mlp1p association with memory gene loops is transcription dependent and is maintained for the duration of the transcriptional memory. Furthermore, deletion of *MLP1* disrupted memory gene loop formation and resulted in loss of transcriptional memory.<sup>81</sup> Gene recruitment to the NPC could enhance memory gene loop formation by acting as a scaffold to promote interactions with both promoter elements and the 3'-end regions of activated genes. Alternatively, the NPC may provide an environment that is conducive for the chromatin reorganization necessary for transcriptional memory as

suggested by the physical interaction of Nup2p and H2A.Z<sup>57</sup>, both of which have been implicated in transcriptional memory of *INO1*.<sup>57, 63</sup>

### Nuclear pore complexes are facilitators of chromatin boundaries

Chromatin boundary elements are stably maintained and inheritable epigenetic regions of specialized chromatin structure that protect adjacent genes from repressive or activating effects of nearby heterochromatin or enhancer elements, respectively (reviewed in<sup>85-87</sup>). Although much progress has been made concerning the role of boundary elements, little is known about their composition or mechanism of action. Surprisingly, components of the nuclear pore basket and nuclear transport machinery were identified in a yeast genetic screen as factors exhibiting robust boundary activity by preventing the spread of heterochromatin into a neighboring active region.<sup>88</sup> Moreover, boundary activity was dependent on physical interaction with the nuclear pore basket, suggesting that the NPC generates a boundary between repressive and active domains.

Nup2p transcends the classical division between the mobile and stationary phases of the transport machinery by cycling on and off the basket of the NPC in a Ran-dependent manner.<sup>74, 75</sup> Deletion of *NUP2* abolishes boundary activity of all NPC associated components tested concomitant with loss of an NPC localized reporter gene.<sup>57, 88</sup> Interestingly, Nup60p, which is responsible for anchoring Nup2p to the NPC, is also required for Nup2p-dependent boundary activity, suggesting that boundary activity of Nup2p requires attachment to NPCs.<sup>57</sup> Proteomic, transcriptomic and genetic studies revealed Nup2p and associated Ran-GEF, Prp20p, physically interact with the boundary proteins histone variant H2A.Z and the chromatin remodeling complex ISW2, and co-isolating chromatin contains epigenetic marks characteristic of boundaries.<sup>57</sup> While a separate proteomic study to identify chromatin complexes at boundary regions failed to detect Prp20p or Nup2p, it did identify multiple Prp20p and Nup2p associated proteins.<sup>89</sup> Given these results a dynamic model of NPC-mediated boundary activity was proposed.<sup>57</sup> In this model the NPC functions as a nexus whereby intranuclear Nup2p associates with chromatin-bound Prp20p at boundary regions, re-association of Nup2p to the nuclear pore basket promotes localization of chromatin to the periphery where the NPC facilitates relocation of chromatin to distinct nuclear subcompartments to stabilize either an active or inactive transcriptional state.

## Disease states associated with altered nuclear envelope function

### Laminopathies

While studies implicating the three-dimensional architecture of the nucleus have significant importance with respect to understanding gene expression, our understanding of these mechanisms promises to have major implications for human health. For example, the nuclear lamina has long been linked to a class of diseases called laminopathies. Laminopathies are a diverse set of heterozygous genetic disorders that include several types of muscular dystrophies, lipodystrophies and premature ageing syndromes and are caused by alterations of the nuclear lamina (e.g. progeria; see Table 1). A single nucleotide substitution, C-to-T at position 1824 of *LMNA* prevents removal of a post-translational lipid modification resulting in a permanently farnesylated form of lamin A, termed progerin, which is irreversibly anchored to the INM.<sup>90</sup> Progerin acts as a dominant negative mutation by disrupting proper lamin polymerization leading to gross nuclear envelope abnormalities and premature ageing as seen in Hutchinson-Gilford progeria syndrome (HGPS; discussed in Box 4).<sup>16</sup> In patient fibroblasts, loss of peripheral heterochromatin is accompanied by epigenetic changes consistent with reduced heterochromatin, specifically a decrease in H3 K9<sup>me3</sup>, a reduction in co-localization of the heterochromatin markers H3 K9<sup>me3</sup> and



heterochromatin protein 1 (HP1), and increased expression of a pericentric heterochromatic region.<sup>91</sup> These results suggest expression of mutant lamins alter the epigenetic organization of pericentric chromatin resulting in changes in gene expression that are likely to have profound impacts on disease progression.

Whereas alterations in lamina structure clearly impact global gene expression through disruption of lamin-chromatin contacts, gene-specific effects also occur through misregulation of lamina associated transcriptional regulators. Studies of the c-Fos and Oct-1 transcription factors (TFs) have demonstrated that sequestration by the lamina prevents these proteins from binding to downstream target genes, providing both an additional method of negative regulation and a mechanism for rapid gene activation.<sup>92-94</sup> Such regulatory mechanisms are likely not limited to c-Fos and Oct-1 as numerous TFs associate with the nuclear lamina including sterol response element binding protein 1 (SREBP1) and retinoblastoma transcriptional regulator (pRb) among others.<sup>95-98</sup> In the case of pRb, interactions with lamin A and lamin C facilitate its peripheral localization.<sup>95, 97</sup> Mutations that alter lamina structure are likely to affect regulation of associated transcription factors leading to changes in expression of target genes. One mechanism by which pRb regulates gene expression is through recruitment of histone methyltransferases, accordingly pRb is required for H3 K9 methylation.<sup>99, 100</sup> This observation suggests that misregulation of pRb may contribute to the epigenetic changes in HGPS cells discussed above. Intriguingly, binding of the transcription factor pRb to the lamina is thought to prevent pRb degradation and promote mesenchymal differentiation of skeletal muscle cells, a tissue often affected by laminopathies.<sup>101</sup>

Like pRb, interactions between SREBP1 and the lamina are important in regulated cellular differentiation. SREBP1 is a transcription factor required for adipocyte differentiation and overexpression of lamin A impairs this process, whereas differentiation occurs more frequently in lamin A/C deficient mouse embryonic fibroblasts (MEFs).<sup>14, 102</sup> Moreover, lamin mutations associated with forms of lipodystrophy displayed reduced binding to SREBP1.<sup>98</sup> Misregulation of transcription factors by structural alterations in the nuclear lamina are likely important factors contributing to disease progression in muscular dystrophies and lipid dystrophies.<sup>101, 103</sup>

### Nucleoporins and cancer

Mutations in four nucleoporins, Nup88, Nup214, Tpr and Nup98, have been associated with cancers of various types including carcinomas, sarcomas and lymphomas (reviewed in<sup>104-107</sup>). The mechanisms underlying these phenotypes are unclear; however, the molecular consequences are similar - genes required for proliferation are up-regulated and genes required for differentiation are down-regulated.

Overexpression of *NUP88* has been reported in multiple tumors including, but not limited to, carcinomas, sarcomas and lymphomas.<sup>108, 109</sup> Nup88 staining of biopsies has been proposed as both a marker of tumor progression and as an indicator of patient prognosis. *NUP88* expression increases during tumorigenesis and high expression levels are associated with poorly differentiated tumors and increased aggressiveness of breast and colorectal carcinomas.<sup>110, 111</sup> Strikingly, *NUP88* overexpression was detected in 76% of ovarian tumors.<sup>112</sup> How might overexpression of *NUP88* lead to cancer? The mechanism is unclear, however there is evidence that Nup88 is required for import of the transcription factor NF- $\kappa$ B and overexpression may alter nuclear trafficking of NF- $\kappa$ B leading to a nuclear accumulation and constitutive NF- $\kappa$ B activity resulting in up-regulation of target genes involved in cell proliferation.<sup>113-115</sup>

Nup214, Tpr and Nup98 are associated with multiple forms of leukemia; chromosome translocation events involving these genes give rise to oncogenic fusion proteins. Fusion of the nucleoporin Tpr with the receptor tyrosine kinase MET creates a cytoplasmic chimeric protein capable of dimerization via the Tpr domain causing receptor kinase activation and subsequent signaling to downstream MAPK and PI3K pathways.<sup>116-118</sup> Loss of negative regulatory elements within the receptor portion of the Tpr-MET fusion permits constitutive signaling generating a transcriptional response promoting cell proliferation (reviewed in<sup>119</sup>). An alternate mechanism leading to receptor dimerization occurs in 6% of patients suffering from T-cell acute lymphoblastic leukemia through fusion of Nup214 and ABL1, encoding the Abl non-receptor tyrosine kinase.<sup>120, 121</sup> Nup214-Abl retains its ability to associate with the cytoplasmic face of the NPC and oncogenic transformation is dependent on interaction with other nucleoporins. Nup214-Abl is unable to self oligomerize; however, the close proximity of multiple chimeric proteins at the NPC is thought to promote constitutive kinase activation inducing cell proliferative pathways including ERK activation and subsequent transcription of the interleukin-2 gene, encoding a mitogen required for T-cell proliferation.<sup>120-122</sup> Although the mechanisms that permit dimerization differ between Tpr-MET and Nup214-Abl, both fusions result in constitutive kinase activation and contribute to tumor progression.

A direct role for NPC components in gene regulation of tumorigenesis was revealed in studies of Nup98. Numerous Nup98 oncogenic fusions have been described (Table 2), each consisting of a partner gene encoding either a DNA or chromatin binding protein and the 5' region of the *NUP98* gene, including *NUP98* promoter elements and an amino acid sequence rich in phenylalanine-glycine (FG) repeats that recruit the histone acetyltransferases CREB binding protein (CBP) and p300 to promote transcription.<sup>123</sup> It is not surprising then, that potent oncogenic Nup98 fusions combine the ability of Nup98's FG region to bind CBP/p300 and transcriptional activators capable of binding DNA, particularly members of the homeodomain family of proteins encoded by the homeobox (*HOX*) genes involved in regulating cell proliferation and differentiation.

In some patients suffering from acute myeloid leukemia (AML) a rare but recurrent translocation event fuses the *NUP98* and *HOXA9* genes.<sup>124, 125</sup> Microarray studies on hematopoietic progenitor cells expressing the *NUP98-HOXA9* fusion indicate that important transcriptional targets of the oncogenic fusion protein are *HOX* genes themselves, and genes functioning in myeloid differentiation, which are up-regulated and down-regulated respectively.<sup>126</sup> The importance of *HOX* genes in nup-mediated cancers are underscored by ChIP studies revealing enrichment of the SET-Nup214 oncogenic fusion protein at *HOXA* gene promoters in patients suffering from T-cell acute lymphoblastic leukemia.<sup>127</sup> Interestingly, although expression of *NUP98-HOXA9* in a mouse model of leukemia resulted in increased proliferation and decreased differentiation of hematopoietic progenitor cells, the onset of leukemia took four months longer than expression of *HOXA9* alone.<sup>128, 129</sup> This result suggests that additional genetic factors are required for full disease progression.

### Systems approaches to identify novel inner nuclear membrane proteins

Despite the fact that numerous diseases are attributable to defects in the nuclear envelope, a molecular understanding of the myriad of functions carried out by the nuclear envelope architecture remains somewhat vague. Defects in the nuclear membrane may contribute to disease through increased fragility of the nucleus under mechanical stress, alterations in chromatin structure, or misregulation of transcription factors, all of which culminate in changes in gene expression. It seems likely that any one of these mechanisms could contribute to disease. To gain a better understanding the nuclear envelope and its contributions to normal and altered gene expression patterns will require further characterization of the nuclear envelope proteome, its dynamics and interactions with

chromatin. Indeed, proteomics studies have begun to illuminate these issues. With the realization of the human genome project, *in silico* based computational approaches have identified a small number of new nuclear envelope components. For example, Nesprin-2 was identified as a gene with high similarity to the human ONM protein Nesprin-1<sup>130</sup>, whereas the mammalian proteins Sun1 and Sun2 were found to be highly homologous to the INM protein Unc84 of *Caenorhabditis elegans*.<sup>131</sup> Interactions between members of the actin-binding Nesprin and lamin-binding Sun family proteins provide a physical connection between the cytoskeleton and nuclear lamina. Importantly, mutations associated with laminopathies disrupt these connections.<sup>132, 133</sup>

Mass spectrometric methods also hold promise for further mechanistic insights. As in any biochemical organelle characterization, a major challenge is to distinguish *bona fide* components of the organelle from contaminants. The nuclear envelope presents a unique complication - the cytoplasmic sheet of the NE is contiguous with the ER. To overcome this problem Schirmer and colleagues<sup>134</sup> used a subtractive hi-throughput shotgun proteomics approach in which nuclear envelope and ER fractions enriched for integral membrane proteins were obtained from rat livers and protein compositions were independently analyzed by multidimensional protein identification technology (MudPIT), which couples tandem mass spectrometry with liquid chromatography to allow analysis of complex biochemical fractions.<sup>135, 136</sup> Proteins identified in both fractions were subtracted from the nuclear envelope fraction, leaving the remaining proteins as possible constituents of the inner nuclear membrane. Using this approach the authors identified 67 novel putative inner nuclear membrane proteins encoding predicted transmembrane domains. Further microscopy analysis confirmed that 8 of 8 candidates tested were localized to the nuclear envelope. Importantly, only 5 of these novel inner membrane proteins did not have an apparent mammalian homolog and, interestingly, 23 genes mapped to chromosome regions linked to a variety of dystrophies with similarity to laminopathies. There is little doubt that molecular characterization of these proteins will lead to a better understanding of the molecular bases of laminopathies and how the 3D architecture of the nucleus and the physical constraints of the NE impact complex control of the genome.

## Future perspectives

It is clear that to understand the complexities of gene regulation we cannot simply view chromatin as a platform onto which regulatory factors bind and release. Indeed, chromatin is highly dynamic and its movement within the nucleus plays a major role in defining, maintaining and switching its activity states. Accordingly, the nuclear architecture itself imparts control on the genome. While we now appreciate these influences, the rules of the game remain elusive. The nuclear periphery does not simply appear to define a state, such as “ON or OFF”; it seems to form part of an organizational domain structure comprised of regions that both promote and prevent gene expression. In addition, studies in yeast reveal that the nuclear pore complex can function to separate active and repressed genes - acting as boundaries between these domains. Moreover, NPCs play a role in transcriptional memory, ensuring cells remember their expression states so that they can retain expression patterns through division and reproduce expression states in future generations.

Yeast has certainly been a tremendously valuable tool to get a foothold into the cell biology of transcriptional control; however, mammalian nuclei are much greater in volume, house more highly organized (hetero)chromatin, dedicate more activity to post-transcriptional control mechanisms, undergo an open mitosis wherein the nuclear envelope completely breaks down at mitosis, and have a significant nuclear lamina structure lining the inner nuclear membrane. We must therefore ask to what extent lessons from yeast can be extended to humans. For example, we presume that in yeast random movements appear to be

sufficient for chromatin to sample large portions of the nuclear volume and that a retention mechanism located at the NE may suffice to restrict movements within the nuclear periphery. But given the large size of mammalian nuclei, do mechanisms exist within the interphase nucleus of mammalian cells that mediate long range gene trafficking? Are there specific (energy dependent) mechanisms at play or does the activity depend on (facilitated) diffusion? In the face of a mitotic disassembly of the organizational structure of the nuclear periphery, what role(s) do the mammalian nuclear periphery play in transcriptional memory, switching transcriptional states, or differentiation? How specific are the influences of the periphery with respect to the expression of gene classes or during development?

While the mechanisms of the phenomena in which nuclear organization influences gene expression are yet to be elucidated, the expanding list of human diseases attributable to nuclear organization based-misregulation reminds us that the answers to these questions are critical.

### BOX 1

#### Systems biology approaches to study the function of NPCs in gene expression

Systems biology has emerged as an important field in understanding complex biological systems. The power of systems biology lies in its ability to integrate high-throughput genetics, functional genomics and proteomics with computational data analyses to identify associations and patterns within complex data sets. Systems biology has been successfully used to understand the function of NPCs in gene regulation. Analysis of global mRNA levels using microarray-based techniques identified the boundary activity of endogenous NPCs.<sup>57</sup> ChIP-CHIP approaches identified specific nuclear pore-promoter interactions, which were further characterized as an early physiological event in transcriptional activation. Furthermore, proteomic methods have been successfully applied to identify protein complexes involved in establishment and maintenance of chromatin boundaries.<sup>89</sup> Recently, computational genomics discovered over 97 promoters with perfect sequence identity to the gene recruitment sequence (GRS) of the *INO1* gene, which functions to target *INO1* to the nuclear periphery during activation.<sup>78</sup>

### BOX 2

#### Identification of genomic lamin binding sites by DamID

A novel protein-genome interaction mapping technology termed “DamID” is based on the construction of a fusion protein consisting of the *Escherichia coli* DNA adenine methyltransferase (Dam) and a chromatin binding protein of interest.<sup>175</sup> Binding of the Dam-fusion to genomic sites permits Dam-mediated adenine methylation of adjacent DNA. DNA isolation, followed by specific amplification of adenine methylated DNA fragments, permits identification of the amplified regions by either direct sequencing or microarray analysis. DamID has been successfully used in *Drosophila* and mammalian systems to assemble genome-wide profiles of DNA sequences bound to the nuclear lamina.<sup>37, 54</sup> These data further demonstrated that transcriptionally inactive genes associate with the lamina.

**BOX 3****Identification of nuclear pore complex-promoter interactions by ChEC**

Chromatin immunoprecipitation (ChIP) has been widely applied to identify protein-chromatin interactions. An alternative, complementary technique suggested to have greater resolution and sensitivity than ChIP is chromatin endogenous cleavage (ChEC).<sup>176</sup> ChEC uses a chimeric protein consisting of micrococcal nuclease (MN) fused to a chromatin binding protein of interest to target associated DNA for cleavage. When expressed in cells, MN fusion protein remains inactive. However, following isolation of cell nuclei, calcium is added to induce nuclease activity resulting in double-strand DNA breaks in the vicinity of the MN-fusion protein binding site. Isolation of DNA and mapping of dsDNA breaks permits identification of cleavage sites and the corresponding binding sites of the MN-fusion protein. Schmid et al. 2006 successfully applied ChEC to map the binding sites of a MN-tagged fusion of the nuclear pore protein Nup2p and determined preferential binding of Nup2p to promoter-proximal regions.<sup>61</sup>

**BOX 4****Hutchinson-Gilford progeria syndrome (HGPS)**

Slowed physical growth and rapid aging is the characteristic symptom of patients suffering from Hutchinson-Gilford progeria syndrome (HGPS), an extremely rare disease affecting 1 in 4 million live births, typically identified at an early age (~12 months). HGPS patients display the following clinical phenotypes: stunted stature, facial-cranial abnormalities, loss of joint mobility, scleroderma, alopecia, loss of subcutaneous body fat (lipodystrophy) but display normal cognitive development. HGPS is fatal with an average life span of 12 years and death attributable to progressive atherosclerosis leading to myocardial infarction or cerebrovascular event. A heterozygous C-to-T substitution at nucleotide 1824 of the lamin A encoding gene *LMNA*, is associated with 90% of all HGPS patients and although it does not affect the amino acid sequence it introduces a cryptic splice site leading to an mRNA lacking 150 nucleotides.<sup>90</sup> The resulting mutant protein, termed progerin, lacks 50 amino acid residues. Removal of the C-terminus also eliminates a cleavage site for the protease ZMPSTE24, preventing this protease from releasing a farnesylated peptide from progerin. Permanent farnesylation of progerin stabilizes its interaction with the NE and is thought to disrupt normal lamin polymerization and function, and potentially manifestation of HGPS.

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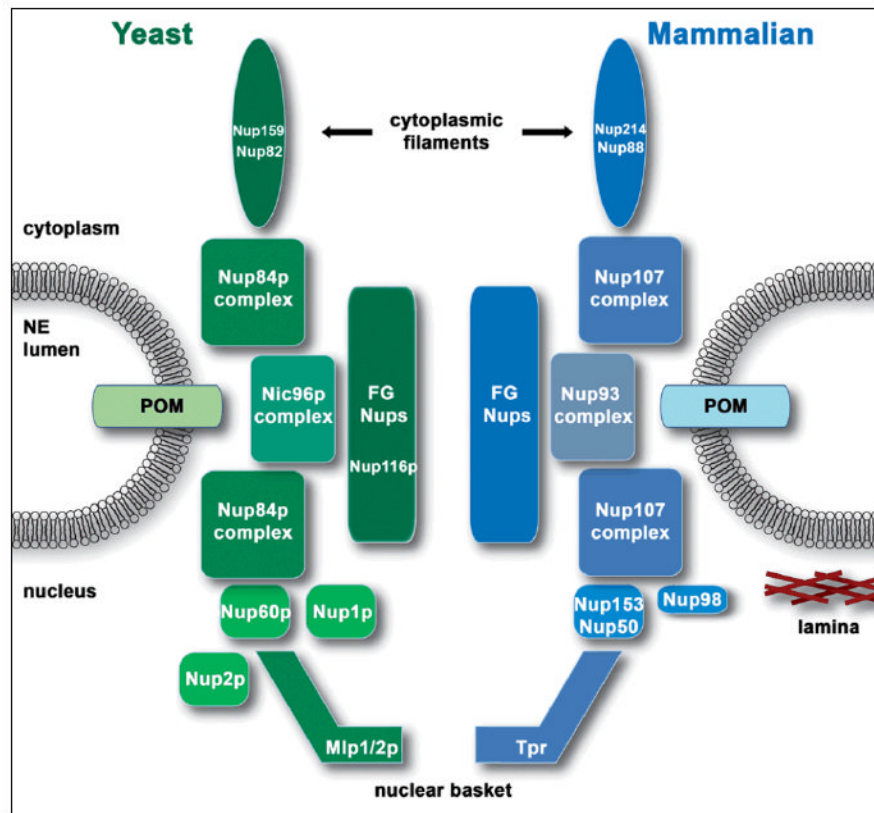
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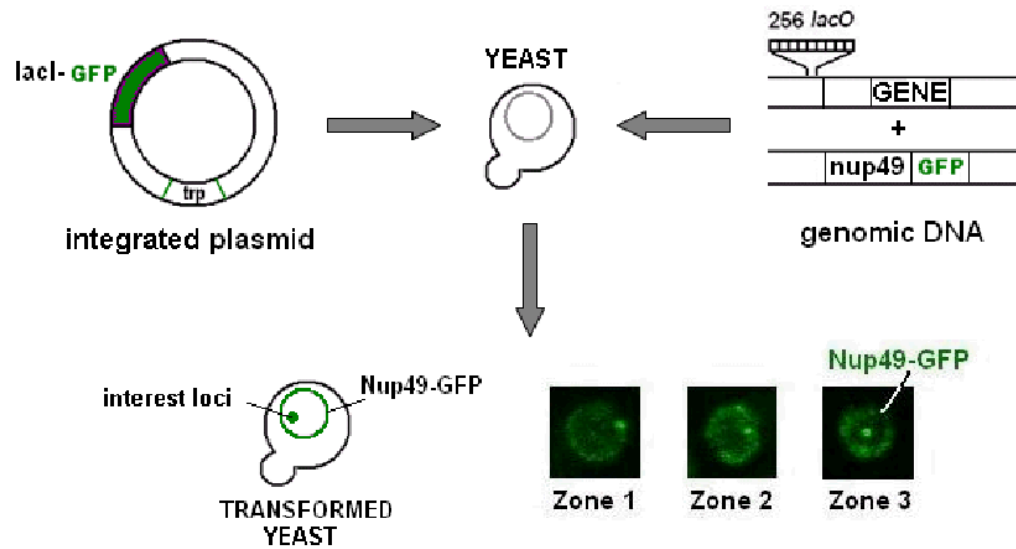
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**FIGURE 1.** Nuclear pore complex. The NPC is a large proteinaceous structure that extends across the nuclear envelope at points where the inner and outer nuclear membranes are fused, creating points of transit between the nucleoplasm and cytoplasm. Interactions with FG-nups lining the central channel facilitates transport of import and export complexes through the NPC. Major sub-complexes of the yeast NPC are shown in green (left side of image) and the corresponding mammalian homologs are shown in blue (right side of image). Positions of specific nucleoporins discussed throughout the text as having roles in gene expression are depicted. POM, pore outer membrane (integral NE proteins that anchor NPCs to the NE; nup, nucleoporin; NE, nuclear envelope; Mlp, myosin like protein; Tpr, translocated promoter region; FG-nups, nucleoporins rich in phenylalanine-glycine repeats.





**FIGURE 2.**

Chromosome tagging. The bacterial *lac* operator (LacO) and *lac* repressor (LacI) system in combination with green fluorescent protein (GFP) has been adapted for visualization of genome organization and dynamics in living cells.<sup>137</sup> The system depends on high expression levels of LacI-GFP, encoded either ectopically on a plasmid or genomically integrated, in conjunction with integration of a tandem array of 256 LacO sequences at the desired genomic locus. LacO sequences are tightly bound by LacI-GFP and are visualized as a bright green focus by fluorescence microscopy (central foci in Zone 3; lower right panel). High expression levels of LacI-GFP bound in relatively close proximity provide a focus that is easily observed with minimal excitation and can be repeatedly visualized with minimal photobleaching, making it ideal for time lapse microscopy in living cells. The addition of a fluorescent marker of the nuclear periphery such as a GFP tagged nucleoporin, Nup49-GFP, and division of the nucleus into three concentric zones of equal area, zone 1-3, aid in determining gene positioning. Chromosome tagging has been successfully used to follow gene loci in both yeast and mammalian systems and has been adapted as a tool to tether genes to the nuclear envelope through the fusion of LacI to an integral inner nuclear membrane protein.<sup>29, 37</sup>

TABLE 1

## Laminopathies

Disease	GENE (protein) affected	Clinical relevance	Refs
Hutchinson-Gilford progeria syndrome	<i>LMNA</i> (lamin A)	Premature ageing, impaired growth, alopecia, loss of subcutaneous body fat, atherosclerosis	90
Emery-Dreifuss muscular dystrophy	<i>LMNA EMD</i> (emerin)	Progressive loss of skeletal muscle, cardiomyopathy, muscle contractures	138, 139
Charot-Marie-Tooth disease	<i>LMNA</i>	Motor defects in lower limbs, foot deformities and loss of tendon reflexes in lower limbs	140
Atypical Werner syndrome	<i>LMNA</i>	Premature ageing apparent in early adults, cataracts, atherosclerosis	141
Limb girdle muscular dystrophy	<i>LMNA</i>	Progressive weakening of shoulder and pelvic muscles, muscle contractures, cardiac arrhythmia	142
Dilated cardiomyopathy	<i>LMNA</i>	Ventricular dilation, cardiac arrhythmia	143
Dunnigan-type familial partial lipodystrophy	<i>LMNA</i>	Loss of adipose tissue, insulin-resistant diabetes, atherosclerosis	144
Mandibuloacral dysplasia	<i>LMNA</i>	Mandibular hypoplasia, delayed cranial development, dental crowding, impaired growth, loss of adipose tissue	145
Restricted dermopathy	<i>LMNA, ZMPSTE24</i>	Microstomia, pulmonary hypoplasia	146, 147
Barraquer-Simons syndrome	<i>LMNB2</i> (lamin B2)	Loss of adipose tissue, more frequent in females (4:1)	148
Autosomal dominant leukodystrophy	<i>LMNB2</i>	Progressive demyelination of central nervous system leading to cerebellar dysfunction	149
Greenberg dysplasia	<i>LBR</i> (lamin B receptor)	Severe skeletal abnormalities, polydactyly, leads to prenatal death	150
Pelger-Huet anomaly	<i>LBR</i>	Heterozygous mutation results in benign hypolobulation of granulocyte nuclei; homozygous mutations lead to skeletal abnormalities	151, 152

TABLE 2

Nucleoporin mutations associated with cancer

Nucleoporin	Fusion partner gene	Partner protein function	refs
<i>NUP88</i>	N/A	NUP88 overexpression	108, 109
<i>NUP98</i>	<i>HOXA9/11/13</i>	Homeobox transcription factors	124, 153, 154
	<i>HOXC11/13</i>	Homeobox transcription factors	155, 156
	<i>HOXD11/13</i>	Homeobox transcription factors	157, 158
	<i>NSD1</i>	Lysine methyltransferase	159
	<i>NSD3</i>	Lysine methyltransferase	160
	<i>JARID1A</i>	Lysine demethylase	161
	<i>PRRX1/2</i>	Homeobox transcription factors	162, 163
	<i>TOP1</i>	Topoisomerase 1	164
	<i>TOP2B</i>	Topoisomerase 2	165
	<i>HHEX</i>	Homeobox transcription factor	166
	<i>SETBP1</i>	SET binding protein 1	167
	<i>RAP1GDS1</i>	Rap1 guanine nucleotide exchange factor	168
	<i>ANKRD28</i>	Ankyrin repeat domain protein 28	169
<i>NUP214</i>	<i>DEK</i>	DNA binding phosphoprotein	170, 171
	<i>SET</i>	Histone chaperone	172
	<i>ABL1</i>	Non-receptor tyrosine kinase	120
<i>TPR</i>	<i>MET</i>	Hepatocyte growth factor receptor	116, 173
	<i>NTRK1</i>	Nerve growth factor receptor tyrosine kinase	174