Histones and histone modifications in perinuclear chromatin anchoring: from yeast to man

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Abstract

It is striking that within a eukaryotic nucleus, the genome can assume specific spatiotemporal distributions that correlate with the cell’s functional states. Cell identity itself is determined by distinct sets of genes that are expressed at a given time. On the level of the individual gene, there is a strong correlation between transcriptional activity and associated histone modifications. Histone modifications act by influencing the recruitment of non-histone proteins and by determining the level of chromatin compaction, transcription factor binding, and transcription elongation. Accumulating evidence also shows that the subnuclear position of a gene or domain correlates with its expression status. Thus, the question arises whether this spatial organization results from or determines a gene’s chromatin status. Although the association of a promoter with the inner nuclear membrane (INM) is neither necessary nor sufficient for repression, the perinuclear sequestration of heterochromatin is nonetheless conserved from yeast to man. How does subnuclear localization influence gene expression? Recent work argues that the common denominator between genome organization and gene expression is the modification of histones and in some cases of histone variants. This provides an important link between local chromatin structure and long-range genome organization and gene expression is the modification of heterochromatin is nonetheless conserved from yeast to man. How does subnuclear localization influence gene expression? Recent work argues that the common denominator between genome organization and gene expression is the modification of histones and in some cases of histone variants. This provides an important link between local chromatin structure and long-range genome organization in interphase cells. In this review, we will evaluate how histones contribute to the latter, and discuss how this might help to regulate genes crucial for cell differentiation.

Keywords CEC-4; histone methylation; inner nuclear membrane; nuclear envelope; nuclear organization

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See the Glossary for abbreviations used in this article.

Changes in heterochromatic histone modifications during cell type commitment

The radial distribution of chromatin changes significantly during cell differentiation (Fig 1). This can be observed during physiological events, for example, during mammalian hematopoiesis [1–4] or Caenorhabditis elegans development [5,6], and occurs during in vitro differentiation events, such as the induction of neuronal cell types from pluripotent embryonic stem cells (ESC) [7–9]. Whereas all cells of an organism contain the same DNA sequence, it is the expression of cell type-specific genes at the right time that determines cell fate and function. Tissue-specific changes in gene expression correlate with changes in histone modifications and de novo CpG methylation [10,11]. Morphologically, differentiation correlates with the appearance of dark-staining foci of heterochromatin. Consistently, compact chromatin domains at the INM or around the nucleolus are less apparent in undifferentiated ES cells than in their differentiated counterparts [3,12,13]. Given that gene activity depends not only on transcription factor availability, but on histone modification and local chromatin structure (reviewed in [14,15]), the sequestration of promoters at the INM may well impact one of these steps, or impose an additional layer of regulation that reinforces stable cell type commitment.

At the heart of the matter are histone modifications, which are known to control local chromatin structure. Indeed, in almost all commonly studied eukaryotic species except budding yeast, heterochromatin is composed of DNA and associated histones bearing specific post-translational modifications. In budding yeast, the absence of histone tail modifications, and explicitly the active removal of histone H4 lysine 16 acetylation (H4K16ac), enables the formation of a heritably silent state characterized by the binding of Sir3 to nucleosomes [16]. In mammals, flies [17–19], and C. elegans [20–22], heterochromatic regions are enriched for histone H3K9 di- and/or trimethylation (H3K9me2 and me3), while in fission yeast H3K9me2 appears to dominate [23]. There are two general classes of heterochromatin in mammalian cells: facultative (regulated) heterochromatin that can be enriched for H3K9me2/3 and H3K27me3, and constitutive heterochromatin that is enriched for H3K9me3 and/or H4K20me3. Whereas H4K20me1 is found in promoters and hyper-active genes, H4K20me3 colocalizes with H3K9me3 in large repeat domains (pericentromeric satellites in mouse) and colocalizes with Heterochromatin protein 1 isotypes (HP1α and HP1β) in differentiated cells [24–27]. In all cases, the resulting repressive chromatin domain restricts access for RNA polymerase or its regulatory factors and, as shown in fission yeast [28,29], may promote transcript degradation. In contrast, the promoters of stably expressed genes harbor histone tail acetylation on histones H3 and H4 and H3K4 trimethylation (H3K4me3).

The Polycomb repressive complex 2 (PRC2) mediates trimethylation of H3K27, which is a further histone modification found on...
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repressed chromatin in differentiated cells [30]. Intriguingly, during zebrafish development and in ESCs, H3K27me3 often coincides with H3K4me3 at poised but transcriptionally inactive promoters [31,32]. Loss of H3K27me3 at some of these promoters later in development correlates with their activation, even though the role of this bivalency in controlling developmental gene expression has been questioned [33,34] (discussed in another review in this series [35]). Indeed, a second Polycomb complex, PRC1, mediates histone H2A lysine 119 monoubiquitination prior to nucleation of an H3K27me3 domain, and FBXL10 (also known as KDM2B, NDY1, JHD1B, and CXXC2), a component of a variant PRC1, wards off CpG methylation at Polycomb target sites [36]. Thus, PRC1 (or its variant forms) may be critical for keeping promoters silent, while ensuring that they are not irreversibly repressed by DNA methylation. Both pathways suggest that Polycomb-mediated repression silences genes in a manner that allows for either subsequent activation or subsequent repression [37,38].

In addition to the accumulation of H3K27me domains, the distribution of H3K9me2 and me3 changes with the establishment of differentiated cell states. The differentiation of hematopoietic progenitor cells, for instance, correlates with increased sequestration of H3K9me3-containing heterochromatin at the INM or around nucleoli, although in these cells global levels of H3K9 methylation seemed not to change [3,39]. Differentiation of stem cells was dependent on G9a, the H3K9 methyltransferase responsible for mono- and dimethylation [3]. Another study reported an increase in levels of H3K9me2 during ESC differentiation, and the concurrent formation of large domains of chromatin bearing H3K9 modifications (LOCKs), which covered one-third of the genome in 100-kb stretches, in differentiated tissues [40]. These different conclusions about H3K9 methylation levels may stem from different modes of quantitation, chromatin isolation [41,42], or from reported cross-reactivity between antibodies recognizing H3K9me2 and me3 [43,44]. Nonetheless, there is a consensus that de-methylation of H3K9 enhances the efficiency of differentiated cell reprogramming [45–48]. Furthermore, the subnuclear distribution of H3K9me3 clearly changes as cells differentiate, with H3K9me3-positive domains being sequestered at the INM. This has been particularly well documented during rodent development [49,50].

The major ligand of H3K9 methylation is a protein called heterochromatin protein 1 (HP1), which has at least three isoforms in mammalian cells and two in C. elegans and Schizosaccharomyces pombe. All HP1 proteins, including the three mammalian isoforms, HP1α, HP1β, and HP1γ, contain an N-terminal chromodomain and a C-terminal chromo-shadow domain. The chromodomain specifically recognizes both H3K9me2 and me3 [51,52], while the
chromo-shadow domain mediates the interaction with other proteins. A spacer domain between them binds RNA. Intriguingly, the different HP1 variants have very distinct roles in the ESC-to-differentiated-cell transition, and not all HP1 binding correlates with heterochromatic gene repression. Indeed, the residence time of HP1 on chromatin is very short [53], and in S. pombe, its RNA binding functions are associated with the restricted spread of a silent domain [28,29].

Confirming this complex role for H3K9me ligands, a recent paper reported that the mammalian HP1β participates both in proper differentiation and in maintaining ESC pluripotency [54]. In ESCs, HP1β is diffusely distributed across the nucleus, unlike in differentiated cells, where it is highly enriched on pericentric heterochromatin. Loss of the HP1β gene in ESCs drove them toward differentiation, while in differentiated cells it lost the opposite effect, increasing the efficiency with which they were reprogrammed into iPSC cells, much like loss of H3K9 methylation [54]. Ablation of HP1α, which binds centromeric satellite sequences in all cells, did not have the same effect. Thus, H3K9 methylation and its ligands can act in diverse ways to control gene expression and cell fate, at least in in vitro systems.

During mammalian embryogenesis, early-stage nuclei are characterized by extensive chromatin remodeling, including dynamic changes in DNA methylation, histone variants, and histone modifications [55]. The core histones H2A, H3.1, and H3.2 exchange rapidly in and out of chromatin in murine pre-implantation embryos, yet this is lost as cells transition from undifferentiated to more determined states [56]. Unlike the case in most somatic cells, pericentric major satellite sequences in early embryos are initially localized around pre-nucleolar bodies and only later become clustered in chromatcenters [57]. Nonetheless, in the maternal genome, these sequences are characterized by classical markers of constitutive heterochromatin, such as H3K9me3, H4K20me3, and HP1β [58,59], which exclude the binding of the Polycomb repressor complex PRC1 [60]. On the paternal genome, on the other hand, a transcriptionally repressed heterochromatic state requires PRC1 and PRC2 proteins, presumably as compensation for the lack of paternal transmission of H3K9me3-marked nucleosomes in sperm [59]. Early paternal heterochromatin is also characterized by high levels of the histone H3 variant H3.3, and overexpression of H3.3 bearing a K27 mutation impairs chromosome segregation and ultimately arrests development [61]. Thus, not only chromatin modifications, but also histone variants are important for heterochromatin formation. The nuclear organization of repetitive DNA is not only highly dynamic in early embryos, but also involves gamete-specific modes of repression.

Accompanying differentiation and the transition from dispersed chromatin to more defined heterochromatic and euchromatic patterns, there is a cell type-specific establishment of late-replicating domains. These coincide with the so-called topologically associating domains (TADs). TADs monitor DNA–DNA interaction probabilities, providing a measure of physical proximity between DNA sequences within the interphase nucleus [62,63]. Not surprisingly, differences in replication timing correlate with different transcription states, and at least a subset of late-replicating domains are associated with the nuclear lamina [63,64].

If we are to prove a causal link between subnuclear position and altered genome function, it will be necessary to show through genetic means that the mechanism that mediates positioning also leads to transcriptional repression and/or late replication. While this link is still elusive in mammalian systems, it has been provided by studies in C. elegans, where histone H3K9 methylation was shown to be essential both for silencing and for the positioning of chromatin at the nuclear periphery [22]. Furthermore, an INM-associated factor, CEC-4, was identified, which specifically recognizes H3K9-methylated chromatin and sequesters it at the nuclear periphery [65]. These studies provide a mechanism to coordinate subnuclear positioning and the establishment of gene repression, as discussed below.

**LADs and chromatin marks that correlate with peripheral attachment**

The nuclear periphery is composed of integral INM proteins and nuclear lamins, collectively called the lamina [66], which binds chromatin in a still poorly defined manner. As mentioned above, chromatin that is associated with the lamina (lamina-associated domains or LADs) is densely packed and enriched for repressive

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**Figure 1. Chromatin distribution changes occur upon cell differentiation.**

In differentiated cells, there are distinct domains of dark-staining heterochromatin at the nuclear periphery and around the nucleolus. Tissue-specific genes are found in heterochromatic zones when repressed and are in euchromatic zones when active. This is true in many species.
histone modifications, most notably H3K9me2/me3 [67,68]. LADs, which monitor even transient association with the nuclear lamina, can cover up to 30% of the genome in mammalian cells, and domains range from 0.1 to 10 Mb in size [67]. While there is a correlation between LADs and LOCKs, the data are derived from disparate cell types and further studies are needed to determine whether LADs and LOCKs are interdependent or whether they can be uncoupled. LAD sequences tend to be gene poor, yet both the border regions of LADs and cell type-specific or variable LADs (vLADs) are significantly enriched for developmentally regulated, cell type-specific genes [9,69]. Such dynamic LAD regions have been previously described as facultative LADs [70]. Nematodes also show a striking correlation between lamin association and repressive histone modifications like H3K9 methylation (H3K9me1, me2, and me3) [20–22]. Thus, in both mammalian cells and worms, H3K9 methylation is enriched in chromatin that associates with the INM [20,67,70,71].

Transgenes that are integrated into the C. elegans genome as multicopy arrays (200–300 tandem copies) accumulate high levels of H3K9me9 and H3K27me3 in a copy number-dependent manner and are quantitatively associated with the INM [5,72]. Consistently, array-borne promoters of ubiquitously expressed genes are transcriptionally repressed both in embryonic and in differentiated somatic cells. When integrated arrays carry tissue-specific promoters, on the other hand, tissue-specific gene activation shifts the domain away from the lamina. This illustrates a general correlation between nuclear position and differentiated cell type gene expression (Fig 1) [5].

A genome-wide RNAi screen was carried out using these large C. elegans gene arrays to identify the mechanisms that link heterochromatin to the INM. Whereas the loss of various chromatin modifiers led to a loss of transcriptional repression, only one RNAi target released the array from the nuclear periphery [22]. This was S-adenosyl methionine synthetase (SAMs), whose loss reduced global histone methylation [22]. The search for downstream effectors of SAMs focused on histone methyltransferases (HMTs), although there was no single HMT whose ablation led to array de-localization. By combining mutants, it could be shown that the elimination of two HMTs, MET-2 and SET-25, compromised INM binding and derepressed the array-borne promoter. MET-2 is an ESET/ SetDB1 homolog that mediates H3K9me1 and me2, and SET-25 is a variant of Suv39h and G9a, which deposits H3K9me3. Elimination of the two enzymes completely eliminated H3K9 methylation in both embryos and larvae and blocked anchoring both of the array and of endogenous repeat-rich heterochromatin in embryos [22].

SET-25, the HMT that deposits the H3K9me3 mark that is necessary for array repression, colocalizes with the silent domain. This colocalization of the HMT, the histone mark, and its reader HPL-1 in perinuclear foci suggested a self-reinforcing mechanism for the establishment and propagation of heterochromatin.

Deposition of H3K9me3 by SET-25 was required for complete array silencing, yet active arrays bearing histone H3K9me1 and me2 were anchored as efficiently as the repressed array [22]. This illustrates a second general principle, that INM association is not sufficient to repress transcription. Moreover, not all transcription events trigger release [5,9,73]. Nonetheless, H3K9 methylation plays an essential role in both events. H3K9me1/me2 can mediate anchoring without silencing (at least in embryos). Anchoring then facilitates trimethylation by SET-25, which remains bound to chromatin by binding H3K9me3. The binding of HP1 homologs and/or LIN-61, an MBT domain protein that also recognizes H3K9me2/me3, also represses transcription but does not anchor the silent domain [22,74]. Finally, we note that although H3K9 methylation is essential for anchoring in early C. elegans embryos, this pathway is not the only heterochromatin-anchoring mechanism that functions in worms. At later developmental stages, such as the first larval stage, sequences that were released from the INM in H3K9 methylation-deficient embryos become re-anchored, although H3K9 methylation remains absent [22]. Thus, alternative pathways for heterochromatin anchoring are induced during terminal differentiation.

A complementary study in mammalian cells has implicated H3K9me2/me3 in positioning chromatin at the nuclear periphery, along with the repressive histone modification H3K27me3 [69]. Harr et al focused on LAD borders, which are enriched for genes that are repressed in a cell type-dependent manner, that bear H3K9me2/3 and H3K27me3, and are critical for cell fate determination [9,69]. Whereas a number of mammalian cell studies have investigated the positioning of repetitive reporters, the system used by the Reddy laboratory instead scored for unique sequences that trigger relocation to the nuclear periphery. The targeting sequences were introduced adjacent to a repetitive lacO array, which was shown to carry H3K9me2/3, that was not sufficient for perinuclear localization in these differentiated murine fibroblasts. However, the integration of sequences derived from vLAD-specific DNA shifted the reporter to the INM [69,75]. Relocation was reduced upon knockdown of the H3K9 methylation-depositing HMT, SUV39H1, or by treatment with a G9a inhibitor. Similarly, Belmont and colleagues showed that the peripheral positioning of a randomly integrated β-globin locus was dependent on both Suv39H-mediated H3K9me3 and G9a-mediated H3K9me2 [76]. G9a inhibition had effects on endogenous sequences as well, reducing the association of LADs with the INM genome-wide [77]. In Harr et al, however, reducing levels of PRC2 to compromise H3K27 methylation and/or treatment with specific EZH2 inhibitors, led to similar reductions in the perinuclear positioning of vLADs [69].

The question arose as to what might recruit PRC2 to vLADs, and therefore, YY1, a transcription factor known to interact with PRC2 [78–81], was targeted to the reporter sequence. Targeted YY1 led to high levels of H3K27me3 on the tagged chromatin and enhanced its association with the INM [69]. Relocation was reduced upon inhibition of the PRC2 catalytic subunit, EZH2, implicating H3K27 methylation in the process. FISH studies confirmed that the localization of vLAD-associated cell type-specific genes at the INM was sensitive to EZH2 inhibition in fibroblasts [69]. In contrast, the removal of PRC2 components mes-3 and mes-6, in C. elegans, did not release heterochromatic arrays from the INM, although it led to their derepression [22]. It should be noted that in worms and mammalian species, most PRC-2- or H3K27me3-positive foci are not found at the nuclear perimeter, suggesting that this mark is not sufficient for relocation [82].

We propose that the combinatorial presence of both H3K9me2/me3 and H3K27me3 may be required to shift locus position in mammalian cells (Fig 2). Consistently, both modifications are found at the borders of cell type-specific LADs [67,69]. Alternatively, a combination of histone marks and the binding of specific
transcription factors may regulate promoter position. Other studies have implicated the transcription factor-mediated recruitment of HDAC3, an INM-associated histone deacetylase, in both peripheral anchoring and repression [71,83,84]. These observations highlight the possibility that histone modifications and other factors could cooperate in a cell type-specific manner to target and maintain heterochromatin at the INM in differentiated mammalian cells (Fig 2). Given that the nuclear envelope proteome is complex and includes many factors with the potential to bind chromatin, we expect that other anchoring factors will function in a cell type- and context-specific manner in differentiated mammalian cells [85–87].

Figure 2. Anchoring chromatin to the nuclear periphery in mammalian cells.
As mammalian cells differentiate, additional domains become associated with the nuclear lamina, called variable LADs (vLADs). These changes between cell types are enriched in cell type-specific genes and are often found at the edges of LADs. (A) vLAD anchoring mechanisms. Borders of vLADs are enriched for both H3K9me2/3 and H3K27me3 and shift to the nuclear periphery in a manner dependent on PRC2 activity as well as on Suv39h1 and G9a. (B) Mechanisms implicated in the anchoring of constitutive/common LADs. They depend on H3K9 methylation deposited by G9a and Suv39h and involve ligands which may include HP1 and other unknown methylation readers. Transcription factor interactions with INM proteins, such as the cKrox (zbtb7b)/HDAC3/Lap2β bridge, may also be relevant for tissue-specific LADs.
Bridging from chromatin to nuclear envelope: anchors for heterochromatin

While genetic manipulations in rodents and human cells have implicated A-type lamins (lamins A and C) and LBR in maintaining heterochromatin at the INM, a direct link between these proteins and any unique marker of peripheral heterochromatin remains elusive. Early studies demonstrated in vitro affinity of LBR for HP1 [88] or for HP1–histone complexes [89], but neither HP1 nor histones are uniquely associated with INM-anchored chromatin domains. Moreover, missing from these early biochemical studies was evidence validating these interactions in living cells. Our laboratory carried out a screen in C. elegans to identify H3K9me ligands that anchor heterochromatin to the INM. We identified an uncharacterized chromodomain protein, CEC-4, which binds mono-, di-, and trimethylated H3K9 and is essential for array anchoring in C. elegans embryos. The loss of CEC-4 phenocopied the array release observed in cells lacking H3K9 methylation, but its loss did not reduce H3K9 methylation levels [65]. CEC-4 is localized to the INM independently of both H3K9 methylation and lamins, forming a perinuclear ring from the earliest stages of embryogenesis through adult tissue differentiation. CEC-4 is displaced from chromatin only when cells undergo mitosis, much like other nuclear lamina proteins [90]. Intriguingly, the localization of CEC-4 itself is not dependent on lamin nor on other known INM proteins, such as emerin, LEM-2, SUN-1, UNC-84, or the LEM domain ligand, BAF-1, and it localizes to the nuclear rim even when expressed in budding yeast [65].

Elimination of CEC-4 in worms released heterochromatin from the periphery, but did not derepress it. This is in contrast to other H3K9me ligands, including the HP1 homolog HPL-2, and LIN-61, whose ablation derepressed the array, but did not affect anchoring (Fig 3) [65]. Remarkably, embryonic gene expression is nearly unchanged despite the loss of CEC-4 under normal laboratory conditions, arguing that INM sequestration of H3K9me is not sufficient nor necessary for gene repression during development. Indeed, cec-4-deficient embryos, like the set-25 met-2 double mutant, were able to differentiate into functional adult worms.

While this indicated that H3K9 methylation and its peripheral sequestration are not essential for carrying out an unperturbed program of development, there were profound differences in outcome when a master regulator of muscle differentiation was induced in synchronized embryos that lack CEC-4. Although cec-4-deficient embryos could induce muscle specification in response to HLH-1 (the MyoD homolog in worms), about 25% of these embryos were unable to restrict expression to the muscle-specific program, as occurs in the wild-type background. An L1 stage gut-specific reporter was expressed in cec-4 mutant embryos, despite the ubiquitous expression of HLH-1, suggesting a further progression of the gut developmental program in cec-4 deficient embryos [65]. It was concluded that perinuclear anchoring of chromatin during development may help to restrict cell differentiation programs by stabilizing the commitment to a specific cell fate. This may be relevant for development when the standard program is derailed, either by environmental or by endogenous perturbations.

Figure 3. Anchoring chromatin to the nuclear periphery in Caenorhabditis elegans.
In C. elegans early embryos, CEC-4 is a H3K9me1, me2, or me3 ligand that mediates anchoring to the nuclear periphery, without necessarily repressing transcription. The H3K9me ligands, HPL1, HPL2, and LIN-61, mediate transcriptional repression by binding H3K9 methylation, but do not anchor chromatin. SET-25 recognizes the H3K9me3-containing chromatin that it creates and together with HP1 homologs and LIN-61 leads to repression. In differentiated cells, alternative anchors may be present.
Alternative pathways tether heterochromatin to the INM in terminally differentiated cells of *C. elegans* larvae and adults in a manner that is independent of H3K9 methylation and CEC-4. H3K27me3 also does not seem to be essential for this anchoring per se, although it may be involved in a combinatorial fashion. In this context, it is useful to note that *C. elegans* does not have self-regenerating and committed stem cells, like those found in higher organisms. Its development and differentiation programs are thus inflexible at the single-cell level. We suspect that peripheral anchoring may be even more important for mammalian differentiation programs, given that committed but undifferentiated states are necessary to ensure tissue replenishment throughout the lifetime of the organism. Finally, although there is no obvious homolog of CEC-4 in mammalian genomes, it is possible that the functions of chromatin binding and perinuclear anchorage are shared among multiple polypeptides and genes in mammals, to allow for combinatorial flexibility in chromatin anchoring.

**Lamins, lamin-associated proteins, and other anchors conserved across species**

Proteins resident at the INM are the most likely candidates to serve as heterochromatin anchors at the INM. A summary of the evidence supporting the role of such candidates is found in Table 1. In mammalian cells, the B- and A-type lamin proteins form a network of intermediate filaments that helps to stabilize the localization of other INM proteins including LAP2, emerin, and MAN1 (the so-called LEM proteins) [91]. In multicellular organisms, LEM domain proteins bind a small, highly dynamic chromatin-associated factor called BAF (barrier to autointegration), which helps mediate nuclear assembly, while lamin A/C binds to a range of transcription factors [92,93]. These lamin-associated factors may contribute to the binding of chromatin regions to the INM, although given that they also associate with active promoters, what is that determines sequence specificity remains unclear [70,94]. The identification and knockdown of 23 INM proteins with transmembrane domains led to the mispositioning of whole chromosomes in mammalian cells [85]. However, each acted selectively on a different set of chromosomes and in a tissue-specific manner [85,95]. Genetic studies also implicate A-type lamins in large-scale chromatin organization [49,96], but to rule out indirect effects, it will be necessary to find mutations that interfere with the recognition of a specific chromatin motif.

Whether acting directly or indirectly, it is clear that lamin A/C, LBR, and Lap2β contribute to the organization of sequences at the INM. Work from the Reddy laboratory showed that lamina-associating sequences are no longer maintained at the nuclear periphery in the absence of lamin A/C [69]. Similarly, in *C. elegans*, the loss of lamin in embryos, or the combined depletion of LEM-2 and MAN-1, led to a partial detachment of large transgene arrays from the INM and stochastically derepressed array-borne promoters [97]. In human cultured cells, it was shown that the factor PRR14 tethered heterochromatin to the nuclear periphery through its association with HP1 and most probably lamin A or C [98]. Whether it binds lamins directly or indirectly, however, is unclear. An extensive study of differentiated cell types by the Solovei laboratory showed that heterochromatin distribution in differentiated rodent tissues depends to varying degrees on two different mechanisms that require LBR and lamin A/C, respectively [49]. Remarkably, in nocturnal rod photoreceptor cells of the retina, which lack both LBR and lamin A/C expression, chromatin has an inverted configuration, such that heterochromatin is found in the center of the nucleus and euchromatin at the periphery. Restoration of LBR, but not of lamin A/C, was sufficient to restore the conventional sequestration of heterochromatin at the INM, and the inverted chromatin configuration could be phenocopied in other differentiated tissues by ablating both LBR and lamin A/C. The authors propose a mechanism whereby LBR mediates peripheral chromatin localization during early development, while lamin A/C becomes more important as cells terminally differentiate [49]. Lamin A/C apparently requires additional proteins or factors to interact with chromatin, which could be factors such as PRR14, transcription factors, or RNA polymerase II regulatory complexes.

LBR is a Tudor domain-containing protein, and through this region, it can interact directly with H4K20me2 *in vitro* [99,100]. It was also reported to bind HP1α and HP1γ [89,101], which are hallmark proteins of heterochromatin [88]. While this sounds promising, HP1α-containing chromocenters are not necessarily perinuclear and HP1γ is bound to many non-peripheral euchromatic loci [102]. Moreover, ablation of HP1α or HP1β in pluripotent or differentiated embryonic stem cells did not alter pericentric heterochromatin organization [54]. Finally, H4K20me2, an *in vitro* ligand of LBR, is distributed broadly across the genome [27], suggesting that it is not significantly enriched in LADs. This does not exclude the possibility that in some situations, LBR-H4K20me2 or LBR-HP1 interactions support chromatin sequestration, but it is clear that this would need to depend on additional marks or interaction domains given the broad distribution of these ligands.

While there is little proof for specific lamin–chromatin recognition, the existing data strongly suggest a role for lamin A/C in conjunction with other INM proteins as a backbone for chromatin sequestration. We note that Zullo *et al* [71] described a role for HDAC3, which deacetylates histones tails, and its interaction with Lap2β, as a tissue-specific chromatin tether (Fig 2). The deacetylation may be a prelude to the deposition of repressive methylation marks. The dependence of peripheral chromatin targeting on histone methylation [22,69,76] and the involvement of INM and lamina proteins suggest that perinuclear anchors that recognize a signature of histone marks and bridge to structural proteins of the INM remain to be discovered in mammals. The characterization of the *C. elegans* anchoring factor, CEC-4, provides an important proof of principle that INM proteins can directly recognize and sequester chromatin bearing specific histone modifications.

**Solving the problem in yeast(s)**

One should not forget that nature has solved the problem of segregating heterochromatic from euchromatic domains multiple times. In budding yeast, telomeres and silent mating-type loci are sequestered at the INM through the interaction of Esc1, a membrane-associated protein, with Sir4, a core component of the repressive SIR complex. There is a second, redundant anchoring pathway that depends on the interaction of Sir4 with yKU, a heterodimer that interacts with the membrane-spanning SUN domain protein Mps3 through a
Table 1. Protein associations implicated in nuclear architecture through direct (d) or indirect (i) interactions.

| Protein | Interaction/association | Method | Interaction | Species/cell type | Effect | References |
|---------|--------------------------|--------|-------------|------------------|--------|------------|
| H3K9me2 and me3 | Heterochromatin formation and differentiation | Microscopy, IF, EM, immunohistochemistry ChIP | i/d | Mammalian (mouse ex vivo expanded MEFs, mouse germ cells, ESC and hematopoietic and retinal cells, cultured mouse fibroblasts), Caenorhabditis elegans, Schizosaccharomyces pombe, Drosophila | Many differentiated cell types show changes in H3K9me2/3 distribution and increased sequestration of H3K9me3-containing heterochromatin at the INM or around nucleoli | [3,17–23,40,50] |
| H3K9me2 and me3 | Subnuclear positioning and repression | Microscopy, IF, immuno-FISH, Dam-ID, shRNA, RNAi, siRNA, drug treatments | d/i | C. elegans embryos, mammalian (cultured mouse NIH3T3 and C57BL/6 fibroblasts) | Essential both for silencing and for perinuclear positioning of chromatin in C. elegans embryos. Contributes to perinuclear anchoring of reporters (mammalian) | [22,69,76] |
| H4K20me3 | H3K9me2/3 and large repeat-rich heterochromatic regions | ChIP, microscopy, IF, and metaphase spreads | i | Mammalian (mouse MEL-F4N cells, erythroid G1E and MEF cells, human HeLa cells, and ex vivo expanded MEFs and mouse trophoblast stem cells) | H4K20me3 colocalizes with H3K9me3 in large repeat domains (pericentromeric), colocalizes with HP1β, in DAPI-dense regions | [25–27] |
| H3K27me3 | Repressed chromatin in differentiated cells and poised inactivation in stem cells | ChIP-seq, sequential ChIP, GMAT (genome-wide mapping technique) | i | Mammalian (human CD4+ T cells, mouse; ES cells, primary lung fibroblasts, immortalized embryonic fibroblasts, C2C12 myoblasts, neuro2a neuroblastoma cells) and zebrafish embryos | PRC2 trimethylates H3K27, which represses developmentally important genes. In ES cells, H3K27me3 can coincide with H3K4me3 at poised but transcriptionally inactive promoters | [30–32] |
| HP1(N-terminal chromodomain) | H3K9me2 and me3 | Binding assays, NMR, in vitro pull-down assays microscopy, IF, co-IP, ChIP-seq, siRNA | d/i | Mammalian (mouse ESC and MEFs, human cancerous HeLa cells, Chinese hamster ovary cells (CHO), mouse oocytes, and mouse sperm) | Roles depend on isoform. The chromodomain recognizes H3K9me2 and me3, the chromo-shadow domain binds proteins. In mammals HP1 variants have distinct roles during ESC differentiation. HP1 binding does not necessarily correlate with repression | [51–54,58,59] |
| Protein | Interaction/association | Method | Interaction | Species/cell type | Effect | References |
|---------|-------------------------|--------|-------------|-------------------|--------|------------|
| Swi6 (HP1 homolog) | RNA as well as H3K9me2 and me3 | ChIP-seq, microscopy (FRAP) | i | S. pombe | Necessary for silent chromatin to which it binds, but also restricts the spread of silent chromatin | [28,29] |
| HPL-2 (HP1 homologous H3K9me2/3 binding factor) | H3K9me3 and H1S-24 (H1) | Microscopy, relocation assay, RNAi | d/i | C. elegans | Works with histone H1 to regulate transcription. Loss depresses but does not delocalize heterochromatin arrays. | [22,65,118] |
| HPL-1 (HP1 homologous H3K9me2/3 binding factor) | H3K9me2/3 | Microscopy, relocation assay, RNAi | d/i | C. elegans | Represses transcription, does not anchor, yet localizes to heterochromatin arrays. RNAi did not derepress test array. | [22,65] |
| Lamin (LMN-1) | Perinuclear large transgene arrays | Relocation assays, RNAi | i | C. elegans | Depletion of the single lamin led to partial detachment of large transgene arrays through development | [97] |
| A-type lamins (LMN A and C) | Peripherally associating test regions and some developmental-specific genes | Microscopy, relocation assays, shRNA | i | Mammalian (mouse C57BL/6 fibroblast cells and primary MEFs) | Reduction in A-type lamins (A and C) released anchored test regions and cell type-specific genes | [69] |
| LEM-2 | Perinuclear chromatin association | ChIP | i | C. elegans | H3K9me2 and me3 are enriched in chromatin recovered by LEM-2 ChIP | [22] |
| Lem2 | Perinuclear chromatin association | ChIP, genetic screen with mutant forms | d/i | S. pombe | Anchors telomeric and centromeric heterochromatin at the INM, and partially ablates repression. Non-overlapping C-terminal MSC and N-terminal LEM domain mediate silencing and anchoring, respectively | [109] |
| LEM-2 and Man-1 | Perinuclear large transgene arrays | Relocation assays, RNAi | i | C. elegans embryos | Combined depletion of LEM-2 and MAN-1 leads to partial detachment of large transgene arrays. | [97] |
| HMTs: MET-2 and SET-25 | H3K9me involvement INM binding and repression | RNAi and microscopy relocation assays | i/d | C. elegans embryos | MET-2, a SETDB1 homolog, mediates mono- and dimethylation, and SET-25 deposits H3K9me3. Elimination of both | [22] |
### Table 1. (continued)

| Protein       | Interaction/association                   | Method                                          | Species/cell type                                                                 | Effect                                                                                                                                                                                                 | References |
|---------------|------------------------------------------|------------------------------------------------|-----------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------|
| HMTs: G9a and Suv39h | H3K9me involvement in perinuclear association | shRNA, siRNA, RNAi, inhibitors, microscopy, IF, immuno-FISH, Dam-iD, ChIP, m^6^A-tracer microscopy/ FISH | Mammalian (mouse C57BL/6 fibroblast cells, mouse NIH 3T3, human Tg3ET cells, Bj−human telomerase reverse transcriptase (hTERT) cells, and HTC75 a human fibrosarcoma cell line) | G9a mediates H3K9 dimethylation and Suv39h mediates H3K9 trimethylation. Peripheral association of test regions, selected endogenous genes and LADs were reduced by reduction in Suv39h and G9a | [69,76,77] |
| CEC-4         | H3K9me1/2/3 and perinuclear association   | Microscopy, relocation assays, RNAi            | C. elegans embryos                                                               | CEC-4 binds mono-, di-, and trimethylated H3K9 and is essential for array anchoring at the nuclear periphery in embryos. Its loss triggers array release as in cells lacking H3K9 methylation, but did not reduce H3K9 methylation levels | [65]       |
| LIN-61        | H3K9me2/3                                 | Microscopy, relocation assay, RNAi             | C. elegans embryos                                                               | Represses transcription, does not anchor to the periphery                                                                                                                                             | [22,65,74] |
| YY1           | PRC2- and H3K27me3-dependent perinuclear association | Microscopy, relocation assay/ChIP, drug inhibition, and shRNA | Mammalian (mouse C57BL/6 fibroblast cells)                                        | YY1 when tethered to chromatin leads to high levels of PRC2-dependent H3K27me3 and peripheral localization. Inhibition of the PRC2 catalytic subunit, EZH2, reduced perinuclear association | [69]       |
| PRR14         | Tethers heterochromatin to the nuclear periphery through HP1 | Microscopy, IF, colocalization siRNA, shRNA    | Mammalian (human HeLa cells and euploid human retinal pigment epithelial 1 [RPE1] cells) | PRR14 tethers heterochromatin to the nuclear periphery in interphase cells binding both HP1 and lamin A/C                                                                                       | [98]       |
| Emerin        | HDAC3                                     | In vitro binding assays, in vivo, co-IPs       | Mammalian (mouse C2C12 myoblasts and emerin^−/−^ mouse myogenic progenitors)       | INM protein, emerin recruits HDAC3 to the nuclear periphery and stimulates its catalytic activity                                                                                                      | [83]       |
### Table 1. (continued)

| Protein       | Interaction/association | Method                                      | Interaction | Species/cell type                                           | Effect                                                                                   | References |
|---------------|-------------------------|---------------------------------------------|-------------|------------------------------------------------------------|-----------------------------------------------------------------------------------------|------------|
| Lap2β         | HDAC3                   | Y2H (in vitro), co-IP (in vivo)             | d           | Mammalian (human osteosarcoma U2OS cells and human lung carcinoma H1299 cells) | Lap2β and HDAC3 interact and lead to the induction of H4 deacetylation.                   | [84]       |
| Zbtb7b (cKrox)| HDAC3/Lap2β             | EMSA, co-IP IF colocalization, shRNA         | d           | Mammalian (mouse NIH 3T3 fibroblasts)                      | Zbtb7b interacts with lamina-associated sequences (LASs) and is predicted to recruit HDAC3 which in turn interacts with Lap2B. Reduction in Zbtb7b and HDAC3 dissociates LASs from the nuclear periphery | [71]       |
| LBR and lamin A/C | heterochromatin localization | Microscopy, immunofISH, IF                | i           | Mammalian (mouse photoreceptor red cells and photoreceptor cells from 39 other mammalian species, mouse ganglion cells and mouse fibroblasts and mouse tissue and skin samples from mice) | Cells lacking LBR and lamin A/C have an inverted chromatin configuration, with heterochromatin in the center of the nucleus and euchromatin at the periphery. Restoration of LBR, but not of lamin A/C, restored normal configuration. | [49,50]   |
| LBR           | H4K20me2                 | In vitro binding assays, co-IP, immunostaining, protein structural analysis | d           | Turkey erythrocytes and E. coli (BL21 [DE3]), mammalian (human cultured cancer cells (HeLa), human pancreatic carcinoma, epithelial-like cell line (PanC1), and transformed human embryonic kidney cells (HEK293)) | The Tudor domain of LBR directly binds H4K20me2. ChiP and co-IPs confirm the interaction of these in vivo, and immunostaining confirms colocalization at the periphery | [99,100]  |
| LBR           | HP1 variants and core histones | In vitro Y2H and co-IP                   | d           | Mammalian (human cultured cancer cells (HeLa) and transformed human embryonic kidney cells (HEK293)) | LBR Tudor domain binds HP1α and HP1γ, which may help tether chromatin to the INM. HP1 forms a quaternary complex with LBR and a subset of core histones *in vitro* | [88,89]  |
| Esc1          | Sir4                    | In vitro, in vivo, Y2H, targeted relocation | d           | S. cerevisiae                                              | Telomeres and silent mating-type loci are sequestered at the INM through Sir4 binding to Esc1, an INM protein, but also through yKu and Mps3 | [103–105] |
telomerase cofactor called Est1 [reviewed in [103]]. Sir4 binds chromatin and DNA non-specifically, but associates tightly with Sir3, which binds the unacetylated histone H4 tail with high specificity. Both interactions are essential for transcriptional silencing (Fig 4A). Thus, in budding yeast, it is the absence of histone marks that promote both repression and anchoring, the former reflecting Sir3 binding to histones and the latter, Sir4’s interaction with INM anchorage sites [16]. Whereas Sir3 and Sir4 function together to silence transcription, Sir4’s anchoring can occur in the absence of Sir3-mediated repression (Fig 4A). That is possible because Sir4 can also be recruited to telomeres by the TG-repeat binding protein Rap1, or by the end-binding protein yKU, to anchor telomeres in the absence of Sir3 [104,105]. Thus, mutations in budding yeast have allowed silencing to be functionally separated from perinuclear tethering, although Sir proteins are involved in both functions (Fig 4A).

Without INM tethering, silencing at telomeres is partially compromised, but mating-type repression persists due to redundant mechanisms for SIR complex nucleation [106]. Intriguingly, the most pronounced defects incurred upon the loss of heterochromatin anchoring in budding yeast were altered telomere length regulation, and increased promiscuity in recombination, possibly due to reduced end protection [106].

In fission yeast, telomeric and centromeric heterochromatin domains are also anchored at the INM, again through multiple mechanisms (Fig 4B). One contributor to this anchorage is Lem2 [107], a member of the conserved family of lamin-associated proteins, that contains both an N-terminal LEM motif, two predicted transmembrane domains as well as an MSC domain [108]. Given that there is only a partial defect in chromatin repression upon the loss of anchoring in S. pombe, Braun and colleagues screened for locus-specific defects in silencing to identify interacting factors, and then, they further analyzed a role for these hits in localization using double and triple mutants [109]. Notably, they found that LEM2 cooperates with the RNAi machinery assembly factor Dsh1 to anchor telomeres and with the centromere factor Csi1 to cluster centromeric heterochromatin at the spindle pole body (SPB, an integral INM structure). The SPB itself is anchored through the SUN domain protein Sad1 (Fig 4B). Two other LAP homologs, Man1 and Ima1, did not show synergistic phenotypes with Dsh1 or Csi1, suggesting that Lem2 is the only LAP homolog involved in silencing in fission yeast [109].

Intriguingly, at S. pombe centromeres, the functions of silencing and anchoring mapped to non-overlapping domains of Lem2. The N-terminal LEM domain promoted centromere tethering, while silencing required the MSC domain [109]. On a mechanistic level, silencing by Lem2 involves the recruitment of the repressor complex SHREC (a Snf2/HDAC repressor complex) to heterochromatin, which contributes not only to repression but also to perinuclear sequestration [110]. Intriguingly, loss of Ctr4, the HMT that methylates H3K9, also displaces silent mating-type loci [111].

At telomeres, S. pombe Lem2 contributes to both repression and tethering, and the functions are not entirely separable [109] (Fig 4B). Recently, Steglich et al [112] demonstrated that the association of telomeres with the INM is reduced in the absence of the nucleosome remodeler Fli3. While single fli3 mutants did not show defects in telomere anchoring, the double mutant bgt3 fli3 showed increased delocalization, which was accompanied by the upregulation of subtelomeric genes. Such defects, however, were mild in comparison with those observed in lem2Δ. Remarkably, the budding yeast homolog of Lem2 (known as Src1 or Heh1) is implicated in the tethering of rDNA to the INM to stabilize the repeated domain, although Src1/Heh1 is dispensable for anchoring SIR-repressed domains [113].

### Summary

Histone H3K9 methylation is essential for the sequestration of chromatin at the nuclear envelope in C. elegans, and most likely this extends to other species, given the conserved role of H3K9 HMTs in heterochromatin formation [114]. The sequestration of
heterochromatin at the INM contributes to the overall organization of the genome into distinct domains called LADs, which are a subset of late-replicating domains and TADs. The cell type-specific roles of various INM components in all species, and the embryo-specific role of worm CEC-4, a chromodomain protein of the INM, suggest that there are differentiation- and developmental state-specific mechanisms that contribute to nuclear organization, rather than one universally conserved mechanism. Given the complexity of the INM proteome and the juxtaposition of tissue-specific transcription factors with the INM and lamin (for further review, see [85,86,115]), we expect that combinatorial mechanisms will anchor heterochromatin in differentiated cells. Functional screens in partially compromised backgrounds will be necessary to dissect the mechanisms that spatially segregate active and inactive genomic domains. Histone modifications are likely to be at the heart of these potentially redundant mechanisms.

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Conflict of interest

The authors declare that they have no conflict of interest.
Sidebar A: Key questions remain to be addressed concerning both the establishment and anchoring of heterochromatin, as well as the function of spatial segregation during differentiation:

(i) Is there a targeted mono- and dimethylation of H3K9 in the cytoplasm and subsequent deposition at specific sequences by a chaperone? Which chaperone is involved?

(ii) Is there active demethylation involved in creating euchromatin?

(iii) What role do HDACs play in the positioning of heterochromatin?

(iv) What bridges chromatin to the nuclear lamina in mammalian cells?

(v) What does BAF play in anchoring?

(vi) Is there a fundamental difference between mechanisms anchoring constitutive and facultative heterochromatin?

(vii) Are there combinatorial signals for cell type-specific anchoring?

(viii) Under what physiological conditions does anchoring become essential for cell type integrity?

(ix) Does mechanical or environmental stress modulate nuclear anchoring?

(x) Is replication timing affected by the same histone marks that determine subnuclear position?

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