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The nuclear envelope — a scaffold for silencing?

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An increasing number of studies indicate that chromosomes are spatially organized in the interphase nucleus and that some genes tend to occupy characteristic zones of the nuclear volume. FISH studies in mammalian cells suggest a differential localization of active and inactive loci, with inactive heterochromatin being largely perinuclear. Recent genome-wide mapping techniques confirm that the nuclear lamina, which lies beneath the nuclear envelope, interacts preferentially with silent genes. To address the functional significance of spatial compartmentation, gain-of-function assays in which chromatin is targeted to the nuclear periphery have now been carried out. Such experiments yielded coherent models in yeast; however, conflicting results in mammalian cells leave it unclear whether these concepts apply to higher organisms. Nevertheless, the recent discovery that evolutionarily conserved inner nuclear membrane proteins support the peripheral anchoring of yeast heterochromatin suggests that certain principles of nuclear organization may hold true from yeast to man.

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Introduction

The cell nucleus contains the essential genetic information of an organism and is responsible for the expression, duplication, and repair of this precious material. Its structure is defined by a double lipid bilayer studded with nuclear pores, which allow macromolecular trafficking in and out of the nuclear compartment. The outer bilayer of the nuclear envelope (NE) closely resembles the endoplasmic reticulum, while the inner nuclear membrane (INM) is specialized to meet the unique nuclear structural and functional needs [1]. In higher eukaryotes, the spherical shape of the nucleus is maintained by a dense network of specialized intermediate filaments, the nuclear lamins. Lamins extend from pore to pore, providing rigidity and a platform for the binding of a large number of lamin-associated proteins and specific genomic domains. A small fraction of lamins are found at internal sites in the nucleus, where again they are thought to organize genomic function [2]. Plants and lower, single-celled organisms do not have nuclear lamins, although other structural proteins of the INM are conserved both in primary structure and in function. In particular, the nuclear pore complex, an elaborate machine for macromolecular transport, harbors many highly conserved proteins [3].

Given this structural conservation it is to be expected that the functions of the NE are also conserved. Indeed, it has long been recognized that dense-staining, transcriptionally silent heterochromatin tends to lie next to the NE or surround the nucleolus, and is specifically excluded from nuclear pores. This has been demonstrated for the repetitive noncoding sequences of vertebrates, and also for silent telomeric chromatin in yeast [4].

Recently, genome-wide techniques have allowed the exploration of sequences and proteins involved in this organization of heterochromatin in higher eukaryotes [5••,6••] as well as in yeast [7]. A number of important questions have emerged from these studies: Does peripheral localization reflect a passive exclusion of heterochromatin from active zones, or do proteins that bind or nucleate heterochromatin have functional anchorage sites at the nuclear periphery? Do all types of silent chromatin bind the NE? Does positioning contribute actively to either heterochromatin establishment or maintenance? Recent reports showing that highly transcribed genes are actively recruited to nuclear pores [7–11] further complicate the picture. How are active and inactive domains kept apart in the nucleus? Nuclear pore attachment has been implicated in providing a boundary function to limit the spread of heterochromatin [12]. This imposes a further question: is localization essential for boundary function or does pore association occur by default?

Correlative evidence has long been used to argue that subnuclear repositioning of genes influences their transcriptional activity. However, such studies cannot directly prove the functional relevance of nuclear architecture. To demonstrate that functional read-outs stem from structural changes one must both perturb nuclear architecture genetically and evaluate gain-of-function assays, for example by tethering chromatin to the nuclear periphery. Such spatial targeting of chromatin was first applied in budding yeast a decade ago [13] and has recently been adapted to experiments in cultured mammalian cells [14••,15••,16••]. Here,

we review these recent experiments and discuss them in view of genetic studies of the nuclear periphery in yeast.

Gene organization along the chromosome arm: functional domains

Chromatin is a contiguous fiber of compact structure and limited flexibility [17]. Therefore, the relocation of a locus to a specific nuclear compartment will inevitably influence the subnuclear position of neighboring genes, encompassing several megabases in mammalian cells [15••]. Consequently, if subnuclear position plays a role in gene regulation, there may be evolutionary pressure toward a linear grouping of coregulated genes along the chromosome arm. A classic example is the linear alignment of the mammalian *HOX* genes, which are arranged in the order of their spatio-temporal activation during limb development [18]. Recent genome-wide analyses indicate that highly transcribed genes are frequently found in clusters [19,20] and that tissue-specific genes are also grouped along the chromosome in higher eukaryotes [21–24]. In *Drosophila*, a computational analysis of 30 occupancy maps extended this observation to chromatin-associated proteins and histone modifications. This study showed that at least 50% of all fly genes are organized in chromosomal domains in which genes bear a similar epigenetic status. Interestingly, the enrichment of common functional annotation keywords (Gene Ontology terms) associated with genes organized in this manner further supported the idea that genes with a common function are grouped into chromosomal units [25•].

Genome-wide studies on nuclear organization

Datasets obtained from microscopic analysis of gene position will never be sufficiently large to test generally whether the transcriptional activity of chromosomal domains correlates with their subnuclear position. However, genome-wide tagging methods such as DamID [26–28] have been used as an alternative method to determine the molecular association of genes with the nuclear lamina. In brief, lamin is expressed as a fusion to the *E. coli dam* methylase, which exclusively methylates adenines. DNA fragments located close to the nuclear lamina are then amplified by a methylation-specific PCR protocol and identified by hybridization to microarrays. This method was recently used to map genomic interactions with B-type lamins in *Drosophila* Kc cells [5••] and human fibroblasts [6••].

In *Drosophila* cells, as well as human fibroblasts, transcriptionally silent genes were found strongly enriched in the lamin-associated fraction. These lamin-bound genes clustered in domains of approximately 500 kb, in agreement with the domain-based model for genome architecture. These domains were depleted for active chromatin marks, were typically flanked by binding sites of the insulator protein CTCF and by CpG islands [6••], and frequently contained coregulated genes [5••].

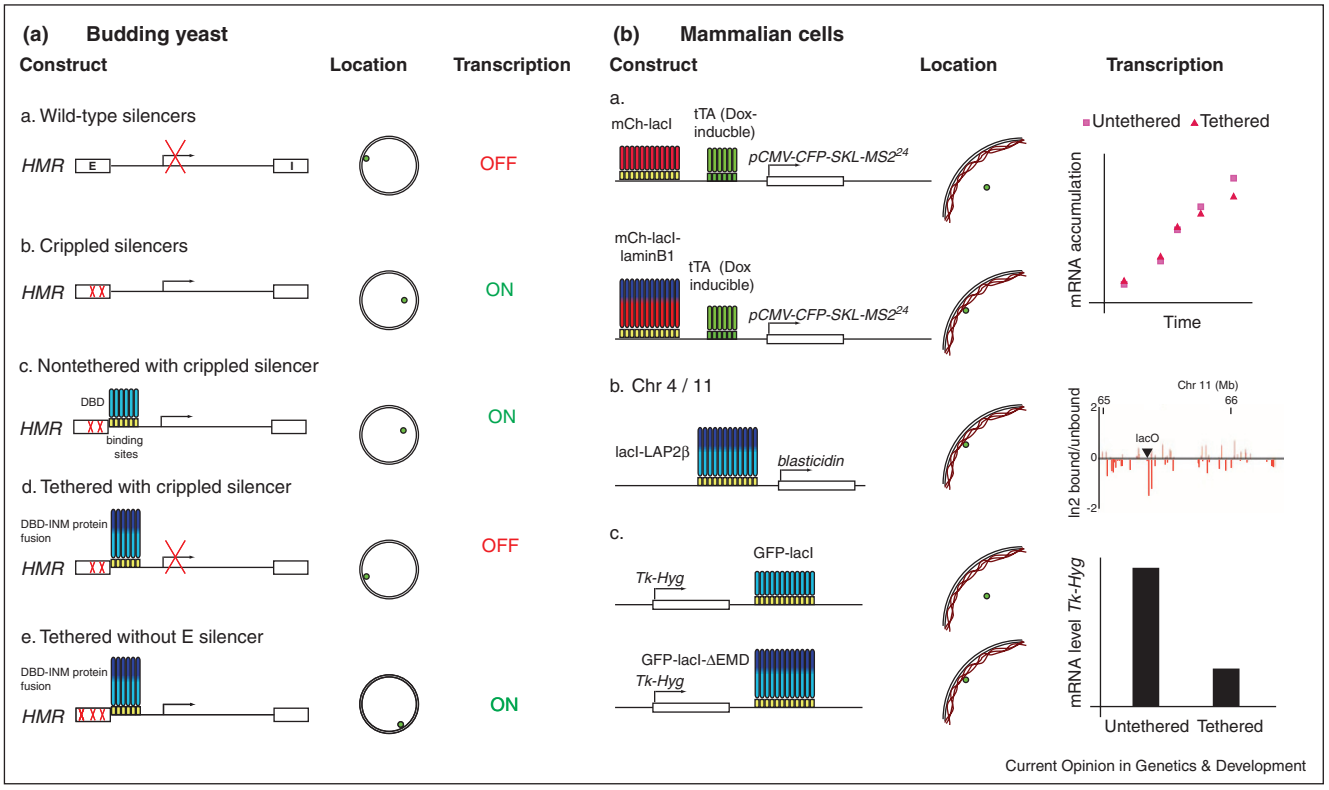
These studies have shown that the association of silent genes with the nuclear periphery is true not only for the handful of genes analyzed by microscopic approaches, but is valid genome-wide. Key questions remaining are what function heterochromatic clustering at the periphery might serve, and which factors determine the peripheral association of silent genes. Studies done with yeast indicate that the structural proteins that form heterochromatin themselves anchor silent loci to the periphery. Indeed, a silenced gene can attach to the periphery even when excised from its genomic context [29]. However, one should not conclude from this that peripheral association is merely a consequence of repression without any functional impact. It is conceivable that the clustering of heterochromatin at the nuclear periphery stabilizes the silent state or helps ensure its epigenetic propagation, for instance by influencing chromatin assembly after replication [30]. The best way to experimentally assess the function of nuclear organization is to modify a gene's subnuclear position. Below, we summarize results obtained using such approaches.

Lessons from genetic manipulation of yeast and flies

Early evidence for a regulatory role of nuclear organization stems from the study of a *Drosophila* translocation mutant allele (bw^D), which contains a block of heterochromatic sequence inserted at the *brown* locus. The mutation causes *brown* to associate with centromeric heterochromatin [31,32]. In animals heterozygous for bw^D, the wild-type allele also associated *in trans* with centromeric heterochromatin owing to the somatic pairing of homologous *Drosophila* chromosomes. Coincident with this association, the wild-type *brown* locus was silenced in a variegated manner. Similarly, silent mating-type loci (*HML* and *HMR*) associate in *trans* with telomeric repeats in yeast [33].

The influence of gene position on the silent mating-type locus *HMR* was assessed more directly in *S. cerevisiae* about 10 years ago [13]. Repression of this locus can be alleviated by the partial disruption of a *cis*-acting silencer element (Figure 1(a)b). However, silencing is restored when *HMR* is artificially recruited to the NE by the expression of a recombinant protein that specifically binds a sequence motif inserted next to *HMR* (Figure 1(a)d). The proposed mechanism for this facilitated silencing was that the perinuclear tethering positioned *HMR* near telomeric foci that sequester the silencing factors (SIR factors) [34]. In support of this concept, it was recently shown that peripheral tethering is unable to restore silencing in a genetic background in which SIR factors are dispersed from foci [35•]. However, placing a gene near SIR foci is not sufficient to cause gene repression, as the *HMR* locus lacking silencer elements is still expressed when recruited to the NE (Figure 1(a)e)

Figure 1



Artificial tethering of chromatin at the nuclear periphery in yeast and mammalian cells. **(a)** Transcription of tethered loci at the nuclear envelope in budding yeast. a. The wild-type silent mating-type locus *HMR*, encoding the mating pheromone is naturally silenced in yeast. The gene is flanked by two silencers, E and I, which target the locus to the nuclear periphery. b. Partial disruption of the E silencer leads to gene expression and delocalization of the locus away from the nuclear envelope. c. Targeting of the locus is achieved by the insertion of binding sites for a DNA binding domain (DBD). Binding of the DBD alone has no effect on gene expression and subnuclear localization. d. Targeting of the DBD (light blue) fused to an inner nuclear membrane protein (dark blue) leads to the relocation of *HMR* to the nuclear envelope. Relocation of a crippled silencer to the nuclear rim can restore silencing. e. This displacement has no effect if the E silencer is entirely removed. This shows the need for *cis*-acting factors for nucleation of silencing. **(b)** Tethering systems used to target chromatin to the nuclear lamina in mammalian cells. a. The system set up by Kumaran and Spector allows one to follow in real time and in live cells inducible transcription and translation of a gene using fluorescent reporters. The locus can be targeted using a fusion between a lac repressor and laminB1. The authors show that the induction rate is similar whether the construct is tethered to the lamina or not. However, tethering decreases the efficiency of induction, since only 70% of the genes can be activated, compared to 90% in the untethered condition. b. A fusion protein between lacI and the lamin associated protein LAP2β is used by Finlan et al. to monitor the effects of peripheral tethering. Expression of a transgenic reporter (blasticidin) is decreased by 20–30%. Transcription of most flanking genes is unaffected, except for three genes which show significantly reduced mRNA levels. c. Reddy *et al.* use a fusion protein with another lamin binding protein, Emerin. A truncated form of Emerin is used that has no transcriptional modulatory function. Tethering a reporter gene (*Hyg*) at the nuclear lamina decreases the transcription level significantly.

[13]. Thus, anchorage near SIR pools at the nuclear envelope facilitates, but is not sufficient for repression.

The influence of peripheral attachment on gene transcription in mammalian cells

Three laboratories have recently adapted such perinuclear targeting experiments to mammalian cell culture systems (Figure 1(b)) [14••,15••,16••]. All three studies made use of cell lines carrying stable genomic integrations of tandem repeats of lac operator (lacO) sites. Through expression of the lacO-binding lacI protein fused directly to Lamin B1 or to the lamin-associated INM proteins Emerin and Lap2β, the lacO arrays and adjacent genes could be tethered to the NE.

The laboratory of David Spector compared the activation dynamics of a doxycyclin-inducible transgene that encodes a fluorescently marked RNA, in the presence or absence of tethering by a LaminB1–lacI fusion (Figure 1(b)a) [14••]. Careful quantification of fluorescence intensity did not reveal any effect of peripheral location on the kinetics of mRNA accumulation in individual cells. However, the fraction of cells in which the transgene could be activated at all was reduced from 90% to 70%.

Similarly, the Bickmore laboratory observed that peripheral tethering using a lacI–Lap2β fusion led to a reduction in the fraction of cells in which a lacO-tagged transgene showed an RNA-FISH signal, and the corresponding

mRNA levels were decreased by 20–30% (Figure 1(b)b) [15••]. More importantly, the expression of most endogenous genes in the neighborhood of the lacO array remained unchanged upon tethering, with the exception of three genes located within 5 Mb of the lacO array whose mRNA levels dropped between 35% and 50%.

The Singh laboratory found that two genes located next to lacO repeats had reduced expression levels when the locus was recruited to the periphery by an Emerin–lacI fusion (Figure 1(b)c) [16••]. Again, the majority of the neighboring genes were unaffected. In contrast to the two other studies, however, the expression of a transgenic reporter located next to the lacO array was robustly reduced by 75%.

In summary, all three studies show that, as in yeast [13,36,37], attachment to the nuclear periphery does not generally preclude transcriptional activity. Nonetheless, the expression of at least some genes is influenced by peripheral tethering. It is likely that the fraction of affected genes is underestimated because of experimental noise that can obscure small expression changes of tethered genes. Moreover, in all cases in which endogenous gene activity was measured, only one of the two homologs was lacO-tagged. Consequently, even complete silencing of a tethered locus would generate only a 50% reduction in expression. Furthermore, the changes in activity may be masked by upregulation of the non-targeted allele through regulatory feedback loops.

It remains to be explored why only a subset of the reporter genes is affected by peripheral attachment. We note that a different peripheral anchor was used in each study, and it is possible that the anchor itself contributes to silencing [14••,15••,16••]. Different anchoring proteins or pathways may function to create distinct microdomains with various levels of transcriptional repression (Figure 2(b)).

Inherent promoter strength could also account for the differential effects of peripheral attachment. For instance, it is well established in yeast that strong promoters block the spread of heterochromatin [38,39]. Similarly, in human cells, active promoters were often found at the edge of lamin-associated chromosomal domains [6••], and in flies it was shown that not all genes respond equally to association with heterochromatic domains [40]. Thus, there is likely to be a complex relationship between gene promoter strength and the effects of tissue-specific factors that influence whether a gene's spatial position affects its expression.

What mechanism confers repression on tethered genes? A simple explanation would be that silencing is not induced by subnuclear relocation, but by the recruitment of transcriptional repressors that are known to bind the INM proteins used for tethering [41]. However, this model was ruled out for Emerin, since the targeting of an Emerin–lacI construct lacking its transmembrane domain failed to induce gene silencing [16••]. Alternatively, gene repression may be stabilized at the nuclear lamina by interaction with other heterochromatic domains in *trans*. In such a model, the NE could serve as a platform for efficient chromatin packing, and its silencing properties would depend on heterochromatin itself.

Finally, a combination of these two models is possible: Lap2β has been shown to directly interact with a histone-deacetylase (HDAC) [42], and inhibition of HDAC activity by Trichostatin A (TSA) was able to relieve Lap2β tethering-induced repression [15••]. In this experiment the tethered locus remained attached at the periphery, whereas in *Drosophila* Kc cells naturally occurring heterochromatic domains were released from the nuclear periphery by the treatment with TSA [5••]. Together these studies suggest a model in which peripheral localization facilitates silencing owing to a peripherally sequestered HDAC activity. At the same time, deacetylated histones themselves may serve as a signal to anchor chromatin at the NE.

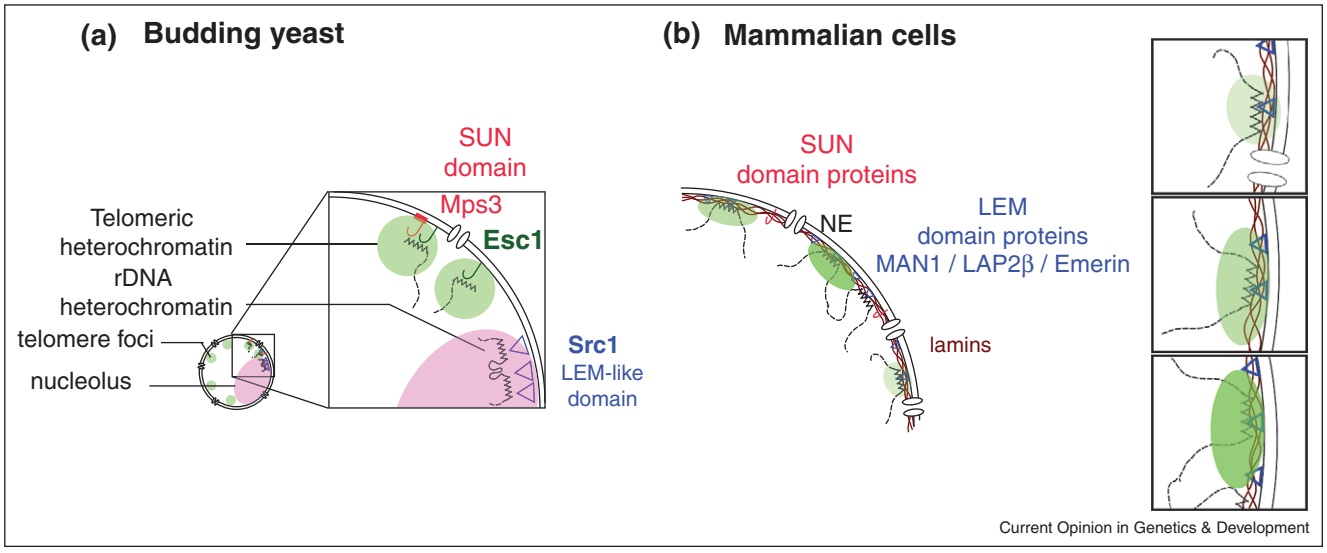
This model is reminiscent of the mechanism suggested for telomere silencing in yeast. In brief, yeast telomeres are maintained at the nuclear periphery by two partially redundant pathways that depend on the DNA-end binding heterodimer Ku70/Ku80, and a structural component of yeast silent chromatin — the silent information regulator Sir4 [43]. In the so-called 'Circe Effect', the nuclear periphery facilitates gene repression by clustering telomeric repeats, which in turn sequester and accumulate the factors required for silencing, including the histone deacetylase Sir2 [30]. Silencing and tethering are thus interdependent: repression promotes attachment, and attachment favors repression as long as telomeric tethers are in place [35•,44].

The yeast nuclear envelope: conserved functions in the absence of lamins

Although an understanding of silencing at the nuclear periphery in yeast is conceptually informative, the mechanistic relevance for mammalian systems has been debated since yeast lack nuclear lamins. Challenging this view, members of evolutionary conserved SUN-domain and LEM-domain INM-protein families have recently been described to play a role in heterochromatin localization and genome stability in budding yeast (Figure 2(a)) [45•,46•,47••].

Members of the SUN-domain family are transmembrane proteins that span the INM and which are anchored in place by binding lamins or other factors. The C-terminal SUN domain of these proteins interacts with Nesprins in the intermembrane space, which forms a link to the cytoplasm through the outer nuclear membrane

Figure 2



SUN and LEM domains proteins organize chromatin at the nuclear periphery in both yeast and mammalian cell nuclei. **(a)** In budding yeast nuclei, where nuclear lamins are absent, telomeres are clustered together at the nuclear periphery (green domains). This depends on Esc1 and the INM SUN-domain protein, Mps3. The nucleolus (red domain) is maintained close to the nuclear envelope by Src1, a LEM-domain family protein. For both proteins, however, no direct interaction with chromatin has been shown to date. **(b)** In mammalian cell nuclei, LEM-domain and SUN-domain containing proteins interact with nuclear lamins and probably indirectly with chromatin (for review, see [50]). LEM-domain proteins may create microdomains at the nuclear periphery. These microdomains may vary in their silencing efficiency which would explain the differences between the three tethering experiments presented in the text. More experiments will have to be carried out using different targeting constructs for tethering of the same reporter to resolve whether anchor specificity or reporter dependent characteristics, such as promoter strength, lead to the variable results.

(ONM), while the N-terminus reaches into the nucleoplasm [48]. Bupp and coworkers have recently shown that even in yeast, where no lamin is present, the SUN-domain protein Mps3 is involved in silent telomere anchoring. The N-terminal domain of Mps3 interacts with Sir4 by pull-down and yeast-two-hybrid experiments, although it is unclear whether this interaction is direct. Via an adjacent domain, called the PAD domain, Sir4 also interacts with the NE protein Esc1 [34]. Nonetheless, in an *mps3* mutant lacking the N-terminal domain, telomeres are partially detached from the periphery, weakly compromising telomeric repression [45•].

Similarly, a role in gene regulation has been shown for a yeast LEM-domain protein by Grund and coworkers. The three types of LEM-domain proteins present in mammalian cells are sequestered at the INM by nuclear lamins. Two of these, Lap2β and Emerin, have an effect on gene expression when tethered to a locus (see above). The yeast protein Src1 (also called Heh1 [47••]) shares homology with the third mammalian LEM protein Man1. Src1 is found at subtelomeric regions, the silent mating-type loci, and the heterochromatin-like rDNA. Gene deletion of *src1* does not affect telomere localization or silencing, although a group of subtelomeric genes is misregulated. This again suggests a role for NE association in gene regulation [46•]. Derepression of subtelomeric genes was also shown to result from telomere delocalization [35•].

Independently, the group of Danesh Moazed reported a function of Src1 at the tandemly repeated yeast rDNA locus [47••]. The deletion of *src1* causes decondensation of the rDNA and partial release of the nucleolus from the nuclear periphery. This release does not affect the silencing of a PolII-transcribed reporter within the rDNA array, which is maintained by the Sir2 HDAC. Instead, the rDNA showed increased recombination rates and changes in array size [47••]. This finding supports another model whereby the sequestration at the nuclear periphery plays a role in the regulation of DNA repair and genomic stability [49•], rather than gene repression. It is not clear whether these two phenomena are linked.

Conclusions

Recent advances have shown that clusters of silent genes associate with the nuclear lamina in mammalian cells [6••]. It is likely that the peripheral localization of heterochromatin is both a cause and a consequence of its repressed state. Careful analysis in mammalian cells using identical reporter systems with a range of peripheral anchors is needed to resolve the conflicts among current results. Nonetheless, these important studies show that, as demonstrated in budding yeast, the positioning of

chromatin at the nuclear periphery can affect gene expression. On the other hand, new studies in yeast reveal another type of perinuclear anchoring that helps to stabilize the genome, rather than conferring transcriptional repression. Whether this also parallels events in higher eukaryotic cells remains to be seen.

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