Review

Mechanisms of heterochromatin subnuclear localization

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Transcriptionally repressed heterochromatin becomes the dominant form of chromatin in most terminally differentiated cells. Moreover, in most cells, at least one class of heterochromatin is positioned adjacent to the nuclear lamina. Recent approaches have addressed the mechanism of heterochromatin localization, in order to determine whether spatial segregation contributes to gene repression. Findings in worms and human cells confirm a role for histone H3K9 methylation in heterochromatin positioning, identifying a modification that is also necessary for gene repression of worm transgenic arrays. These pathways appear to be conserved, although mutations in mammalian cells have weaker effects, possibly due to redundancy in positioning mechanisms. We propose a general model in which perinuclear anchoring is linked to an epigenetic propagation of the heterochromatic state, through histone modification.

Segregating chromatin types in the interphase nucleus

Increasing evidence indicates that models depicting chromatin as a linear template for transcription do not capture all of its regulatory potential, thereby suggesting that higher-order chromatin folding may help regulate gene expression. Although our understanding of higher-order chromatin structure at the molecular level is poor, chromatin organization *in vivo* has been intensively studied at a macroscopic level. Such approaches have yielded strong evidence for a nonrandom distribution of chromatin in interphase nuclei (reviewed in [1,2]).

A recurrent theme in nuclear organization is the spatial segregation of chromatin of different types. Domains with high transcriptional activity are either in the nuclear center or in rare cases, associated with nuclear pores, whereas transcriptionally silent domains are associated with the nuclear periphery or the nucleolus. Although some differentiated cells – such as the rod photoreceptor cells of nocturnal rodents [3] – have a different nuclear organization, the conventional distribution of active and inactive domains is found in both differentiated and undifferentiated cells, from a range of species from yeast to humans. Fundamentally distinct experimental approaches including chromosome conformation capture (i.e., 4C and Hi-C), DNA adenosine methyltransferase Identification (DamID), chromatin immunoprecipitation (ChIP), and fluorescence microscopy, contribute to this picture of nuclear structure [4,5]. Recent studies have coupled these approaches with genetic screens to identify mechanisms that position heterochromatin in the nucleus. In this review we summarize the latest advances in this field.

Microscopic visualization of subnuclear compartments

The most direct evidence showing a spatial segregation of chromatin types into distinct subnuclear compartments comes from fluorescence and electron microscopy (reviewed in [5,6]). Image analysis shows that the clustering of silent chromatin into DNA-dense foci at the nuclear envelope (NE) and around the nucleolus is a feature of nearly every cell type. By both fluorescence *in situ* hybridization (FISH) and live microscopy, specific loci have been shown to be associated with peripheral heterochromatic compartments when silent, and to relocate away from these compartments upon activation [7–14]. This is particularly evident for genes expressed in a tissue-specific or cell-type-specific manner [15]. In some cases, co-regulated, tissue-specific promoters cluster in assemblies that contain multiple active RNA polymerases [16,17].

Although correlated with transcription, the movement of genes away from heterochromatin and the NE is unlikely to result directly from gene expression, because promoters of housekeeping genes can be highly transcribed and remain peripherally localized [7,18–20]. Indeed, the tethering of promoters to the NE has been shown to promote, but not be sufficient to repress promoters in yeast; repression requires that positioning is coupled with sequences that nucleate repressor binding [21,22].

The nuclear lamina: a scaffold for silent chromatin

The visualization of genetic loci in living cells by time-lapse microscopy has shown that chromatin undergoes constant Brownian-like motion in the nucleus within restricted domains [23,24]. Interestingly, silent genes that are associated with the nuclear periphery are more constrained in their movement than active genes in the nuclear center [23,25]. This indicates that genes at the nuclear periphery are likely to be molecularly tethered, though in a reversible manner, to a relatively immobile nuclear structure. Two structures have been proposed to provide this scaffold function. One is the nuclear lamina, which lies directly under the nuclear membrane in all

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Keywords: heterochromatin; nuclear envelope; nuclear lamina; H3K9 methylation; *Caenorhabditis elegans*.

0968-0004/$ – see front matter
 http://dx.doi.org/10.1016/j.tibs.2013.04.004

eukaryotes that undergo an open mitosis. The nuclear lamina or its associated proteins are thought to interact with silent chromatin (reviewed in [4,26]). The second peripheral platform is the nuclear pore complex, which binds active genes, in addition to ensuring nuclear transport (reviewed in [27]). Stress-induced genes, in particular, tend to colocalize with nuclear pores both prior to and after their induction in organisms as diverse as yeast, flies, and worms [28–31]. This review examines the spatial organization of heritably silent heterochromatin, which is excluded from nuclear pores and enriched at the nuclear lamina.

The nuclear lamina: a simple backbone and complex network of associated proteins

The central components of the nuclear lamina are the lamin proteins themselves, which form a meshwork of intermediate filaments on the inner face of the NE [26,32]. An increasing number of proteins is reported to contact the lamins, directly or indirectly, forming a complex network of interdependent proteins at the nuclear periphery (reviewed in [33]). Interestingly, several NE components can directly bind chromatin. For example, the chromatin-bound, barrier-to-autointegration factor (BAF) bridges to at least three lamin-associated transmembrane proteins, namely Emerin, MAN1 (target of MAN autoimmune antibody 1) and LAP2β (lamina-associated polypeptide 2β, reviewed in [34]), whereas the lamin B receptor (LBR) is reported to bind either H3K9me2/3 through heterochromatin protein 1 (HP1) [35], or histone H4K20 methylation through its C-terminal Tudor domain [36] (Figure 1). Lamins have also been shown to interact directly with histones and DNA *in vitro* [37,38], although it is unclear if these low-affinity interactions are relevant *in vivo*. Nonetheless, the role of lamins as a scaffold for perinuclear chromatin is supported by genetic data: loci that are preferentially NE-associated relocate to the nuclear center upon the depletion of lamins [39–41], and/or downregulation of LBR or lamin A/C [3].

Further evidence for a role of lamins in perinuclear gene targeting stems from DamID and ChIP experiments [42–46]. In a pioneering study, Pickersgill *et al*. reported the specific lamin interaction of 500 genes in *Drosophila* Kc cells [42–46]. As expected, lamin association correlated with low gene expression levels, and with the absence of H3K4me3 and H4K16ac chromatin marks. However, lamin interactions were also shown to be dynamic, and distinct changes in lamin binding were observed upon induction of differentiation by treatment with the developmental hormone ecdysone. The resulting loss of lamin interaction correlated with gene activation, and, conversely, gene repression was accompanied by a gain in lamin binding [46]. Subsequent genome-wide studies in mouse and human cells showed that these characteristics were conserved in mammals [44,45] and consistent results have also been obtained from ChIP and DamID studies in *Caenorhabditis elegans* [43,47]. The longer-range resolution of genome-wide studies shows that lamin-associated domains (LADs) form large clusters in *cis* that often span multiple genes, and extend up to 10 Mb in size [45].

Lamin attachment affects large-scale chromatin folding and DNA–DNA contacts

LADs are flanked by boundary elements enriched for CTCF (CCCTC binding factor) sites [45]. The correlation of LAD boundaries and CTCF sites was explored further in a study that mapped CTCF-associated chromatin loops by chromatin interaction analysis with paired-end tag sequencing (ChIA-PET) [48,49]. CTCF-mediated loops were strongly enriched at the border of LADs [42], whereas the domains themselves were refractory to loop formation. This suggested an intimate link between the anchoring of chromatin to the nuclear lamina, and an inhibition of long-range DNA contacts mediated by CTCF [50]. This

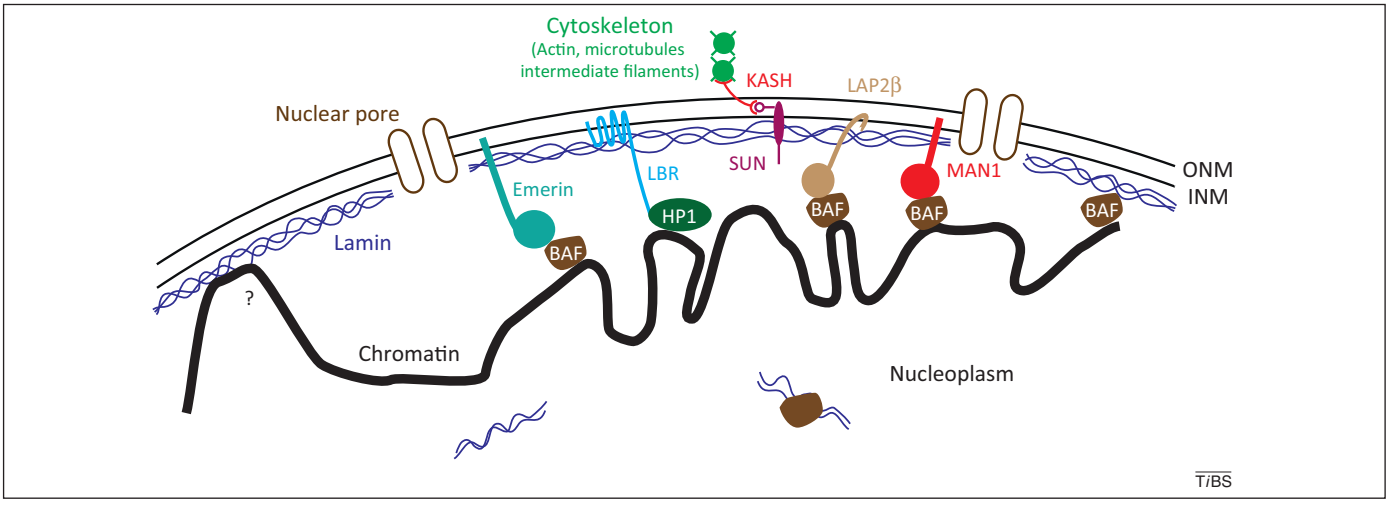

Figure 1. Schematic view of the nuclear envelope (NE). Lamins form a meshwork of intermediate filament proteins underlying the inner nuclear membrane (INM), where they interact with several distinct classes of transmembrane proteins. One class contains the NE-associated proteins Emerin, LAP2β (lamina-associated polypeptide 2β) and MAN1 (target of MAN autoimmune antibody 1), which interact via a conserved LEM (Lamina-associated-polypeptide, Emerin and MAN1)-related domain with the small protein barrier-to-autointegration factor (BAF), which has affinity for chromatin. A second example is the lamin B receptor (LBR), which contains seven transmembrane domains that span the INM. It interacts directly with the chromatin binding protein HP1 or in mammals with H4K20me through a Tudor domain. Finally, SUN (Sad1–UNC84 homology) domain proteins span the INM, and interact with lamins on the nucleoplasmic side and with KASH (Klarsicht/ANC-1/Syne Homology) domain proteins in the perinuclear space. KASH proteins (called Nesprins in human) span the outer nuclear membrane (ONM) and contact the cytoskeleton (adapted from [33,72]). Up to 10% of lamins and BAF are also found in the nucleoplasm in some cells.

important factor was also found enriched around euchromatic islands within large heterochromatic domains [48], suggesting that it may also control the spread of lamin association in *cis*.

Independent of CTCF function, Hi-C contact maps indicate that attachment to the nuclear lamina reduces the likelihood that a locus will interact with non-LAD domains in the genome. At least at the resolution of 1 Mb, DNA contacts within LADs, or within non-LAD regions, were both substantially more frequent than interactions between lamin-bound and non-bound loci [51]. It remains to be determined whether this trend will also hold true at higher resolution. If it does, then lamin-association may repress gene expression by physically impairing the interaction of a promoter with its distant enhancer element. This is consistent with the finding that long-range chromatin organization helps ensure that an ectopically integrated enhancer element activates exclusively the target genes located in its spatial proximity [52].

Testing the function of perinuclear chromatin anchoring

These correlative studies strengthen the hypothesis that long-range chromatin architecture influences gene expression. To test this model, however, one must interfere with chromatin organization and study the consequences of this perturbation on gene expression [6]. One approach has been to tether a chromosomal locus that is normally internally located, to the nuclear periphery through the targeting of an anchorage protein to the locus. Inspired by the extensive use of such targeted fusions in budding yeast [21,22,53], three groups have targeted mammalian genomic loci to the NE in an inducible manner in cultured cells [18,19,54]. These studies indicate that artificial relocation of chromatin to the periphery can favor gene repression, at least for some promoters. NE-tethering of transgenes also enhanced their repression in *Drosophila* [55], although in every case, some promoters seemed to be indifferent to the influence of subnuclear localization [7,18,19,54]. Consistent with this, work from budding yeast convincingly demonstrated that the enhanced silencing promoted by NE tethering was entirely dependent on the enrichment of the silent information regulatory (SIR) complex in perinuclear foci, which themselves are created by the tethering and clustering of SIR-bound telomeres [21,22]. Thus, it is not position alone, but juxtaposition to other silent domains that is necessary for NE-mediated repression.

How is endogenous chromatin addressed to the nuclear periphery? Sequence analysis of mammalian and fruit fly LADs did not reveal significantly enriched sequence motifs [4], suggesting that chromatin positioning is not directly determined by consensus sequences. However, LADs were strongly correlated with specific histone modifications [44,45]: H3K9me2 was enriched throughout 80% of all LADs [44,56], and the boundaries of lamin-bound regions had high levels of H3K27me3 [44,45].

A similar correlation between perinuclear chromatin anchoring and a specific histone modification was reported for *C. elegans*. In this case, the chromatin associated with the nuclear envelope was determined by ChIP using antibodies raised against LEM-2, a lamin interacting transmembrane protein homologous to human MAN1 (Figure 4, [43]). On all worm chromosomes, LEM-2 domains were consistently located on the extremities of chromosomes (chromosome arms), whereas chromosomal cores were enriched for highly conserved, essential genes [57], and were depleted from with the nuclear envelope. All three methylation states of H3K9 (me1, me2, and me3) showed a common pattern: they were depleted from the chromosome center and enriched in the distal LEM-2 domains [58]. Importantly, the same distribution was observed for repetitive sequences [59].

Recent work from the Gasser laboratory has exploited repetitive transgenes in *C. elegans* as an experimental tool to probe perinuclear chromatin anchoring in embryos genetically. Like heterochromatic chromosome arms, large integrated transgene arrays were shown to accumulate high levels of histone modifications that are characteristic for silent chromatin (H3K9me3 and H3K27me3) [40]. The repressive marks were deposited in a copy- number-dependent manner: transgene arrays with high repeat copy number ($\geq$250) carried methylated histone H3, while smaller insertions of 50–70 tandem copies did not. The accumulation of repressive chromatin marks also correlated with the subnuclear distribution of these arrays. In embryonic nuclei, small arrays were randomly distributed throughout the nuclear space, whereas the large, transcriptionally repressed arrays of identical sequence composition were peripheral, suggesting a correlation between histone modifications and peripheral chromatin localization [7,40]. Depletion of lamin from *C. elegans* embryos by RNAi led to a partial detachment of the large transgene arrays from the NE, and genetic ablation of lamin led to a stochastic de-repression of array-borne promoters [39]. These genetic experiments reinforced the correlation suggested by mammalian cell studies: the nuclear lamina sequesters repressed chromatin enriched in heterochromatic histone modifications.

Role of H3K9 methylation in anchoring chromatin to the NE

Although this correlation is striking, it does not argue for a causal relation between histone modification and chromatin position. To this end, a genome-wide RNAi screen for factors involved in the peripheral tethering of heterochromatic transgene arrays was performed in *C. elegans*. Intriguingly, many factors were identified whose loss resulted in transgene array de-repression, but only one RNAi target in the *C. elegans* genome led to the release of the heterochromatic array from the nuclear periphery, along with array de-repression [47]. This target was a pair of closely related genes that encode S-adenosyl methionine (SAM) synthetase (*sams-3* and *sams-4*). Reduced levels of SAM synthetases reduced histone methylation globally, leading to both transcriptional upregulation and the release of the array from the NE. Many other RNAi clones led to de-repression without affecting localization, confirming again that transcription alone does not necessarily release chromatin from the NE.

A systematic analysis of histone methyltransferases (HMTs) was carried out in order to identify the modifications and targets relevant for the heterochromatin anchoring. There was no single HMT whose loss led to

heterochromatin release, yet the combined elimination of the genes encoding two histone H3K9 HMTs, MET-2 and SET-25, mimicked the loss of SAM synthetases. The MET-2 enzyme is the worm ESET/SetDB1 homolog that mediates mono- and dimethylation of H3K9, whereas SET-25 is a distant variant of Suv39h and G9a, which deposits H3K9me3. Combined depletion of the two HMTs abrogated the perinuclear attachment, not only of heterochromatic transgenes, but also for the endogenous NE-associated chromatin on chromosome arms, which is similarly enriched for H3K9 methylation in wild type cells [47].

Methylation of H3K9 independently triggers both repression and anchoring

The MET-2 and SET-25 HMTs have been shown to target H3K9 in a consecutive fashion: MET-2 mediates mono- and dimethylation only, whereas SET-25 is the only worm enzyme that can trimethylate H3K9, thus replacing both G9a and Suv39h enzymes in mammalian cells. SET-25 colocalizes with its own product, H3K9me3, in perinuclear heterochromatin foci. Importantly, sequestration of SET-25 in these foci requires H3K9me3, but does not simply reflect affinity of its catalytic domain for its product. The colocalization of this terminal HMT with the mark that mediates both repression and localization suggests an autonomous, self-reinforcing mechanism for the establishment and propagation of repeat-rich heterochromatin. It generates a nuclear subcompartment enriched for the H3K9 HMT that is needed to repress transcription (Figure 2). We note that loss of SET-25 alone derepresses the transgene array, but does not lead to array delocalization. Chromatin in the *set-25* mutant contains histone H3K9me1 or me2, which is sufficient to anchor it to the NE. Thus, one can conclude that me1 and me2 states of H3K9 are sufficient for perinuclear anchoring, whereas further modification, generating H3K9me3, is necessary for transcriptional repression. These results show causality between histone H3K9 methylation and anchorage.

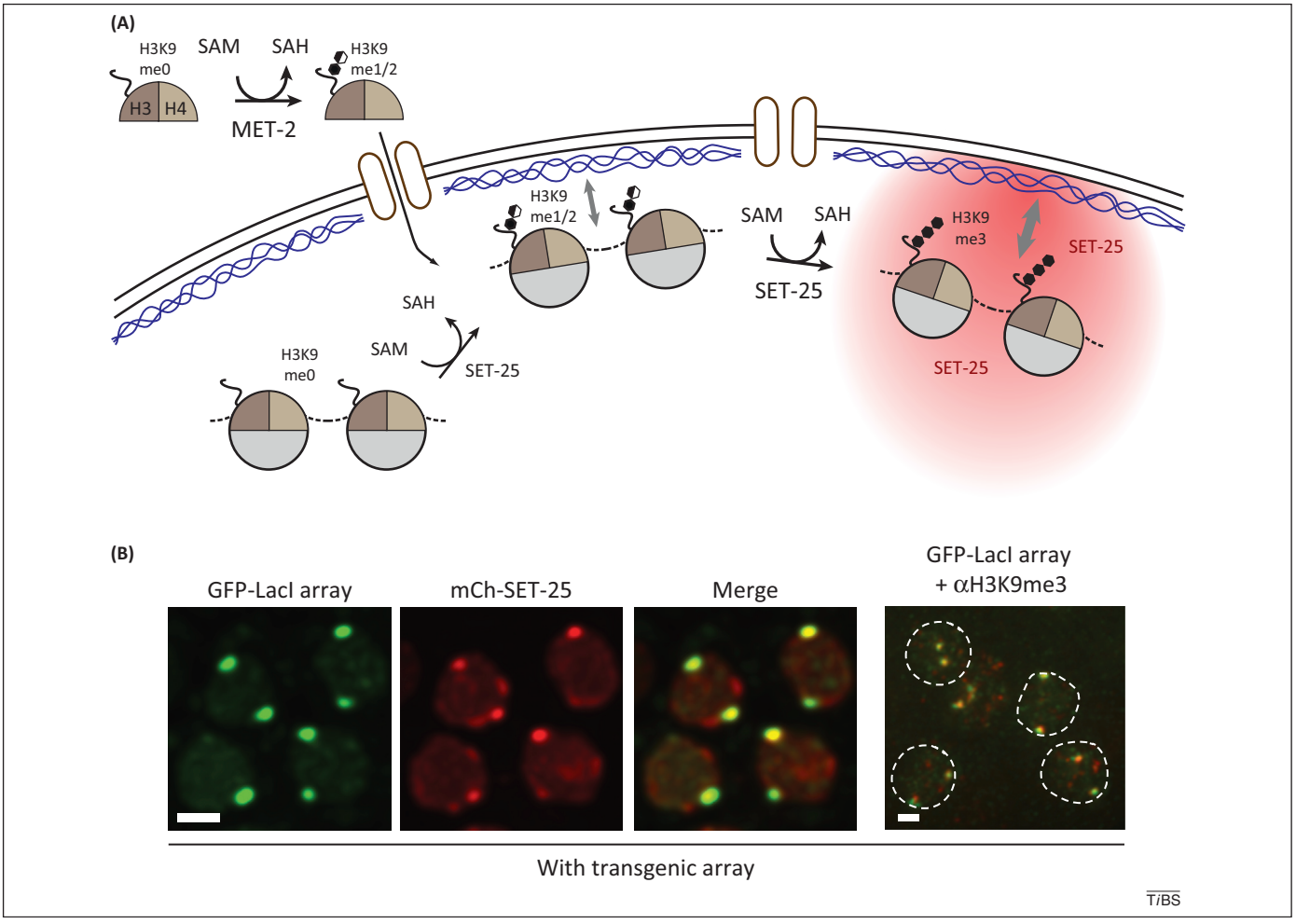

Figure 2. Histone H3K9 stepwise methylation by two histone methyltransferases (HMTs) is a trigger for peripheral anchoring of heterochromatin. **(A)** In *Caenorhabditis elegans* histone H3K9 is methylated in a stepwise manner by the HMTs MET-2 (homolog of ESET (ERG-associated protein with SET domain)/SetDB1 (SET domain, bifurcated 1)) and SET-25, the unique H3K9 trimethytransferase in worms. MET-2 is localized in the cytoplasm where it mono- and dimethylates H3K9 prior to histone incorporation into nucleosomes. S-Adenosyl methionine (SAM) is the universal donor for all methylation reactions. Nuclear SET-25 is sufficient to form H3K9me1/2 and me3, but its efficacy of trimethylation is strongly enhanced when H3K9me1/2 is provided by MET-2. H3K9me1/2 initiates chromatin anchoring, and H3K9me3 is required for complete array silencing and enhances peripheral array attachment. The SET-25 enzyme accumulates in perinuclear foci (red diffuse zone) in a manner dependent on its own reaction product, H3K9me3. The model is modified from [47]. **(B)** The terminal H3K9 methyl transferase SET-25 colocalizes with heterochromatic transgene arrays in *C. elegans* nuclei. The SET-25 protein was tagged N-terminally with mCherry and expressed in *C. elegans* embryos under control of the ubiquitously active *his-72* promoter. Shown are representative nuclei of 50- to 100-cell embryos in which the mCherry–SET-25 accumulates on the large GFP-tagged heterochromatic array at the nuclear periphery. In the fourth panel, antibodies that recognize the methylated H3K9 histone residue are shown to colocalize with the GFP-tagged heterochromatic array in embryos. Dashed circles indicate outlines of nuclei. Images are reproduced from [47]. Scale bars = 5 μm.

They also underscore the link between generation of a silent domain and its NE anchorage: the anchorage-competent H3K9me1/me2 states are essential to generate H3K9me3, which confers transcriptional repression.

The question arises why so many chromatin-modifying pathways affect silencing, whereas only one affects the anchorage of heterochromatin, at least in the *C. elegans* embryo. From the systematic RNAi screen it is obvious that not only perturbation of histone methylation on H3K9, but also loss of the Polycomb-deposited mark (H3K27me) or of H3K36 methylation, leads to a partial loss of transcriptional repression [47]. This may either indicate that the repression of repetitive transgene arrays requires more than the H3K9me/HP1 pathway, or else that there are indirect effects that arise from genome-wide alterations of nucleosome modifications. Nonetheless, no other major methylation-acceptor site on any of the core histones is altered by loss of SET-25 and MET-2 [47]. Whereas this makes it clear that anchoring requires H3K9 methylation and can occur independently of transcriptional repression, it does not exclude that other modifications contribute to the H3K9-dependent anchoring signal. In short, histone H3K9 methylation is necessary, but may not be sufficient for heterochromatin tethering.

To what extent is histone H3K9 methylation a conserved signal for NE anchoring of chromatin? In a study that addressed the general stability of lamin-associated domains in a human fibrosarcoma cell line (HT1080), Kind and colleagues found that lamin-associated chromatin was enriched for histone H3K9me2, but not H3K9me3; consistent with earlier work [44,45,60]. The stochastic reassociation of heterochromatin with the nuclear lamina after mitosis was shown to correlate with H3K9me2, and the ablation of G9a, one of several H3K9 HMTs in mammalian cells, reduced but did not eliminate the perinuclear positioning of LADs after mitosis [60]. H3K9 methylation is not entirely absent in these cells, like it is in the *set-25 met-2* double mutant of *C. elegans*, therefore, it is difficult to know whether mammalian LADs absolutely require H3K9 methylation for peripheral positioning. The parallel between this and the worm system is nonetheless striking.

Importantly, even in *C. elegans* the H3K9 methylation-mediated anchoring pathway is not the only mechanism that tethers chromatin at the nuclear envelope. For instance, in differentiated tissues of later developmental stages (e.g., the first larval stage), repeat arrays become re-anchored in the *set-25 met-2* double mutant, even though H3K9 methylation is still undetectable [47]. This argues for an alternative, differentiation-induced pathway of heterochromatin anchoring. Such redundancy is reminiscent of observations made for tissues deficient for either lamin A/C or LBR in mice [3]. A thorough study of single and double mutants for these two NE proteins has shown that LBR is the crucial anchorage site in early stages of mouse cell differentiation, whereas lamin A/C replaces or supplements its role in terminally differentiated cells [3]. We propose that redundant anchoring pathways exist for heterochromatin universally, although they may act sequentially or cell-type specifically during organismal development. In worms, a putative second anchorage pathway appears to become functional late in cellular differentiation, and seems to be H3K9me independent. Although methylation on H4K20 may be the signal recognized by LBR in differentiating mouse tissues [36], the signals for the alternative anchoring pathway in worms, like the motif recognized by the mammalian lamin A/C on chromatin, are unknown.

How does H3K9me target chromatin to the NE?

Although it is possible that H3K9me could bind lamin or a lamin-associated protein directly, the more likely scenario in the worm is that this interaction is mediated by other factors that contain a methylation-recognition motif, such as a Chromo-, Tudor-, or MBT (malignant brain tumor) domain. Intriguingly, array anchoring does not require the *C. elegans* homologs of HP1 (*hpl-1* and *hpl-2*), which are well-characterized ligands of methylated H3K9, nor does it require LIN-61, an MBT protein that binds H3K9me2/3 *in vitro* [61]. Moreover, the closest LBR homolog in worms lacks the C-terminal Tudor domain, which mediates histone binding in the mammalian LBR [36]. Because the worm genome-wide screen did not detect a chromodomain-containing protein whose loss led to both de-repression and de-localization of the heterochromatic array, we speculate that the anchoring factor may tether without being required for gene repression.

It has been shown in mammalian cells that the loss of tethering does not always lead to de-repression of tissue-specific genes. Indeed, the loss of the perinuclear anchors lamin A/C or LBR in transgenic mice had opposite effects on the expression of muscle-related genes in differentiating myoblasts [3]. Specifically, loss of lamin A/C reduced expression of 41 muscle-related genes, whereas loss of LBR led to a slightly increased expression of the same set of genes, but only during the initial stages of differentiation [3]. The same gene ablations had little or no effect on the expression profile in terminally differentiated muscle, arguing that NE positioning may be most critical during cell-type determination, and not in terminally differentiated states.

H3K9 methylation along the chromosome: centromeres and perinuclear heterochromatin

In mammals, as in fission yeast and flies, H3K9 methylation is found primarily on centromeric repeats or on heritably silent promoters [62]. Yet, centromeric heterochromatin is not always found at the NE, particularly in mammalian cultured cells. Given that *C. elegans* chromosomes are holocentric and lack centromeric satellite DNA, their H3K9-methylated chromatin is restricted to the terminal 3–5 Mb of each chromosome arm [58]. These domains coimmunoprecipitate with the nuclear envelope component LEM-2 [43] and associate with the nuclear lamina in a manner sensitive to *set-25 met-2* mutations [47]. The effect of HMT mutations in mammalian cells is less clear. Although G9a ablation reduces the stochastic reassociation of LADs with the NE in a human cell line [60], mutation of the H3K9 HMT G9a alone does not significantly affect the perinuclear localization of LADs in mouse embryonic stem cells, despite a strong reduction in peripheral H3K9me2 levels [63]. Moreover, loss of both Suv39h isozymes in mouse cells, which compromises levels of H3K9me3 in heterochromatin, does not alter the

organization of centromeres [64]. In contrast, we note that the loss of LBR, which preferentially binds histone H4K20 methylation, perturbs the peripheral localization of heterochromatin in differentiating mouse cells [36].

These results raise the question whether other repressive histone marks compensate for the absence of G9a methylation, at least in some cell types. The persistence of subnuclear chromatin organization could stem from a compensatory function of H3K9me1, which accumulates in Suv39h1/2 double mutants [64]. Recent evidence shows that in mammals, H3K9me1 is deposited by two H3K9-specific mono-methyltransferases of the positive regulatory domain (PRDM) family, PRDM3 and PRDM16 [65]. Downregulation of both enzymes leads to a loss of H3K9me1, which also reduces H3K9 me2 and me3. This loss leads to a dispersal of centromeric foci, an accumulation of major satellite transcripts, and perturbed ultrastructure of the nuclear lamina [65]. Centromeric heterochromatin is not lamin bound, but it may affect long-range interactions between other heterochromatic domains. In other words, the loss of one heterochromatin compartment may feed back to influence the integrity of the lamin/LAD compartment, generating the described phenotypes indirectly. Clearly, further work is required to understand the relation between centromeric heterochromatin and lamin-associated domains in mammalian cells.

The kinetics and spatial organization of H3K9 methylation

If we are to understand how peripheral positioning affects gene repression, it is crucial to address the question of how and where the different steps of H3K9 methylation occur. The first steps of the stepwise methylation of H3K9 (i.e., me1/2 deposition by MET-2, or me1 by ESET, PRDM3 or PRDM16) appear to take place at least partially in the cytoplasm, on histones prior to their incorporation into nucleosomes. Consistently, the MET-2 enzyme is primarily cytoplasmic in worm embryos [47], PRDM3 and PRDM16 are partially cytoplasmic in cultured cells [65], as is SetDB1/ESET [66]. Indeed, one third of non-nucleosomal histone H3 in HeLa cells bears H3K9me1, and is presumably cytoplasmic [66,67]. Thus, there must either be a selective deposition of this population of methylated H3/H4 dimers into heterochromatin during its replication, or else a selective removal of H3K9me1 in domains destined to be euchromatic. Deposition of the H3/H4 dimer bearing H3K9me1 or me2 appears not only to be necessary, but may indeed be sufficient, to confer NE attachment on replicating heterochromatin (Figure 3).

The subsequent steps of modification, which are required for transcriptional repression, are generally carried out by another set of enzymes, for example, SET-25 in worms, and either G9a or Suv39h in mammalian cells. All three of these H3K9 HMTs are recruited to chromatin by the marks they deposit: indeed, SET-25 is highly enriched on silent heterochromatic arrays [47]. This may trigger terminal modification of neighboring nucleosomes and a spread of the repressive chromatin mark. Good evidence supports the existence of a similar mechanism for H3K27me3 propagation by Polycomb repressive complex (PRC)2 [68], for H3K9me3 spreading in fission yeast by Clr4 [69] and for the maintenance of H3K9me3 at centromeric repeats in mammals by Suv39h [70,71]. The worm HMT SET-25 appears to follow suit, although in contrast to Suv39h, which is recruited to methylated H3K9 by the chromodomain protein HP1 [70,71], SET-25 does not depend on the worm HP1 homolog, HPL-1, to associate with the silent array. Indeed, SET-25 has little or no sequence homology to Suv39h outside its SET domain and lacks a chromodomain. Nonetheless, the enrichment of SET-25 in foci of silent heterochromatin suggests a means to propagate the repressed state by replicating the template chromatin in the SET-25-enriched subcompartment.

A self-reinforcing mechanism to sequester silent chromatin at the nuclear periphery

Given that H3K9 methylation is a trigger for both perinuclear chromatin anchoring and silencing, the two

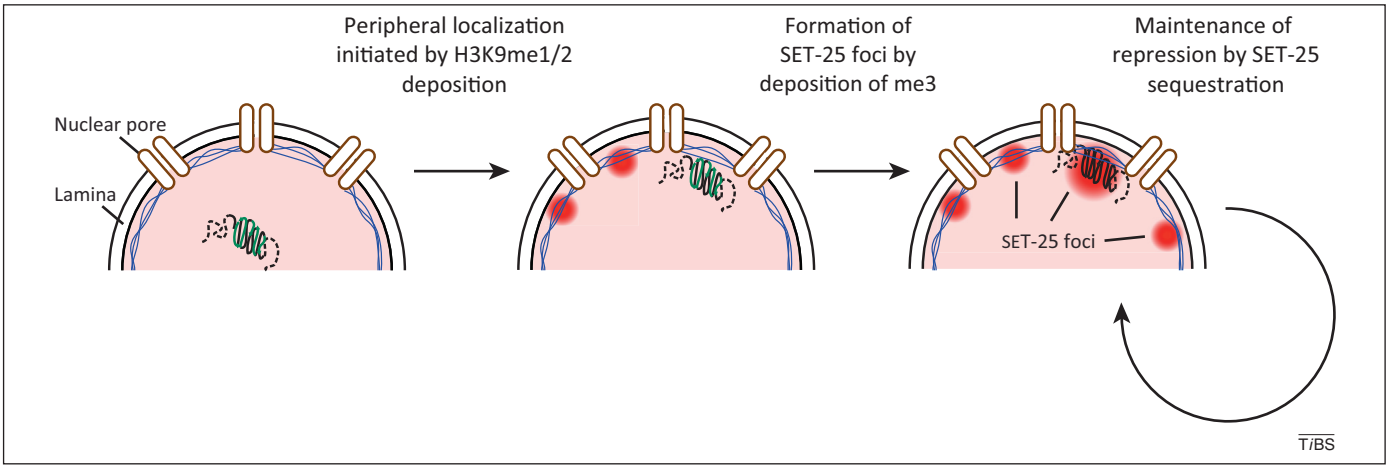

Figure 3. Model for a self-reinforcing mechanism for perinuclear anchoring and heterochromatin silencing. In wild type conditions, chromosome arms acquire H3K9 mono- and dimethylation and relocate to the nuclear periphery where they encounter existing loci of silent heterochromatin. These foci contain both H3K9me3 and the histone methyltransferase SET-25 which deposits this mark, and which remains associated with heterochromatin after terminal methylation. The deposition of me1 and me2 does not silence (green line), but allows peripheral localization, where trimethylation occurs and transcription is repressed (black line). The sequestration of SET-25 in foci of existing heterochromatin increases H3K9 trimethyltransferase activity locally, enhancing methylation on nucleosomes brought into its proximity by the affinity of H3K9 mono- and dimethylation for the nuclear envelope. Thus, histone methylation promotes both anchoring and repression, first in a sequential manner and later as a feedback loop.

functions are linked at the molecular level. They are also spatially linked, because SET-25, which mediates the repressive trimethylation, is enriched in a subcompartment containing heterochromatin at the NE. Retention and localization of a repression-mediating enzyme at the NE may lead to a circular, reinforcing mechanism for the segregation of active and inactive chromatin domains (Figure 3).

A similar mechanism has been demonstrated for SIR-mediated silencing in budding yeast, in which peripheral anchoring is mediated by the chromatin-bound Sir4 protein. Sir4 is similarly required as an integral component of silent chromatin [53]. The fact that yeast chromatin can be tethered prior to full inactivation and that the recruitment of a chromosomal locus to the nuclear envelope can promote its repression (reviewed in [72]), argues that peripheral localization of heterochromatin serves both a repressive and a propagation function. The finding in worms that chromatin marks carry subnuclear positioning information, opens the door to the discovery of new components of the anchoring pathway.

Concluding remarks

The most interesting insight to arise from the study of worm heterochromatin, is that that *C. elegans* embryos can develop into viable and differentiated worms despite being completely devoid of H3K9 methylation. This suggests either that transcription factors alone orchestrate the changes in gene expression that are necessary for cell differentiation, or that other chromatin marks carry out the task of H3K9 methylation, to repress certain genes during development. The H3K9 methylation-deficient worms progressively lose fertility over several generations, and are stress sensitive. As in higher eukaryotes, transposon or viral element instability appears to increase [73–75] (P. Zeller *et al.*, unpublished). Nonetheless, the viability of this strain means that *C. elegans* provides a system in which to study a complex organism without H3K9 methylation. It will also be an ideal system in which to study the re-establishment of heterochromatin, upon controlled reinduction of the SET-25 or MET-2 HMTs. As we identify the essential molecules and pathways that contribute to and substitute for heterochromatin, we may also learn what heterochromatin really contributes to the life of a cell.

Acknowledgments

We thank the Gasser laboratory for stimulating conversations. The Gasser laboratory is supported by the European Union Network of Excellence 'Epigenome', the Novartis Research Foundation, and the Swiss National Center for Competence in Research, 'Frontiers in Genetics'.

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