Repetitive Transgenes in *C. elegans* Accumulate Heterochromatic Marks and Are Sequestered at the Nuclear Envelope in a Copy-Number- and Lamin-Dependent Manner

B.D. Towbin, P. Meister, B.L. Pike, and S.M. Gasser
Friedrich Miescher Institute for Biomedical Research, CH-4058 Basel, Switzerland
Correspondence: susan.gasser@fmi.ch

Chromatin is nonrandomly distributed in nuclear space, yet the functional significance of this remains unclear. Here, we make use of transgenes carrying developmentally regulated promoters to study subnuclear gene positioning during the development of *Caenorhabditis elegans*. We found that small transgenes (copy number ≤50) are randomly distributed in early embryonic nuclei, independent of promoter activity. However, in differentiated tissues, these same transgenes occupied specific subnuclear positions: When promoters are repressed, transgenes are found at the nuclear periphery, whereas active, developmentally regulated promoters are enriched in the nuclear core. The absence of specific transgene positioning in embryonic nuclei does not reflect an absence of proteins that mediate perinuclear sequestration: Embryonic nuclei are able to sequester much larger transgene arrays (copy number 300–500) at the periphery. This size-dependent peripheral positioning of gene arrays in early embryos correlates with the accumulation of heterochromatic marks (H3K9me3 and H3K27me3) on large arrays. Interestingly, depletion of nuclear lamina components caused release of arrays from the nuclear envelope and interfered with their efficient silencing. Our results suggest that developmentally silenced chromatin binds the nuclear lamina in a manner correlated with the deposition of heterochromatic marks. Peripheral sequestration of chromatin may, in turn, support the maintenance of silencing.

Differentiation of a pluripotent stem cell into specified cell types is a tightly regulated process that requires multiple layers of control. Cell identity is first set by the activation of tissue-specific gene programs. The expression status of a gene can subsequently be maintained by a local modification of chromatin structure through posttranslational modification of the histone octamer and binding of additional chromatin-associated factors (Boyer et al. 2006; Mohn and Schübeler 2009). Local modulation of chromatin structure may change a gene's accessibility to DNA-binding factors and to the transcriptional machinery and hence regulate its activity.

It has been postulated that the higher-order structure of chromatin, and its spatial organization within the nucleus, may also contribute to gene regulation, possibly independent of local chromatin structure and accessibility (Wilson and Berk 2010). In particular, the spatial separation of inactive loci from active genes in nuclear subdomains is thought to support their efficient repression by locally increasing the concentration of silencing factors (Gasser et al. 2004). Conversely, spatial separation may prevent promiscuous silencing that would occur by silencing factors bound to normally active genes (Taddei et al. 2009). Although specific genes have been found to interact with the nuclear pore and subnuclear bodies, the best evidence for a functional role of subnuclear organization in gene regulation concerns the association of facultative heterochromatin to the perinuclear lamina.

The nuclear lamina consists of a dense meshwork of intermediate filament proteins (lamins) that mechanically maintain the spherical shape of the nucleus (Dechat et al. 2008). In addition to this morphological function, the nuclear lamina serves as a binding platform for various factors involved in transcriptional regulation and chromatin metabolism, supporting the idea of a regulatory role of perinuclear gene localization (Taddei et al. 2004). Moreover, scientific interest in the association of chromatin with the nuclear lamina has been fostered by the discovery of a large number of genetic diseases (laminopathies) that are caused by mutations in lamin itself or other structural components of the nuclear envelope (Verstraeten et al. 2007).

Historically, one addressed the functional implications of chromatin association to the nuclear lamina by describing the nature of lamina-bound chromatin. The earliest of these studies used electron microscopy to show that electron-dense material is in close proximity to the nuclear lamina (Busch 1966). Subsequently, a number of specific loci have been shown by fluorescent in situ hybridization (FISH) to specifically associate with the nuclear periphery when inactive (Spector 2003; Deniaud and Bickmore 2009). Most recently, a method termed DamID was applied to characterize the nature of DNA bound to the nuclear lamina on a genome-wide level in *Drosophila* embryos (Pickersgill et al. 2006) as well as human cell culture systems (Guelen et al. 2008; Peric-Hupkes et al. 2010). Together, these studies demonstrated that chromatin bound to the nuclear lamina is generally inactive and carries post-translational histone modifications characteristic of silent loci.

Complementing this correlative approach, several laboratories have artificially targeted specific loci to the nuclear periphery (Andrulis et al. 1998; Finlan et al. 2008; Kumaran and Spector 2008; Reddy et al. 2008). In all cases, at least a mild reduction in transcriptional activity was observed for some promoters, although the magnitude of repression varied from study to study and seemed to depend on the nature of the targeted promoter (for discussion, see Towbin et al. 2009). Tethering of chromosome segments to the nuclear envelope could identify a function of the nuclear lamina in repressing genes that are artificially recruited to it. However, these experiments did not address how genes are recruited to the nuclear envelope without an artificial anchor and whether positioning might be used during development to control cell-type-specific expression.

Here, we tracked the subnuclear position of transgenes that contain developmentally regulated promoters during development of the nematode *Caenorhabditis elegans*. We find that subnuclear localization of low-copy transgenes carrying developmentally regulated promoters depends on a cell's differentiation state: In early embryos, transgenes were randomly distributed through nuclear space, independent of promoter activity. In contrast, following differentiation, active and inactive promoters were spatially separated. Transgenes with inactive promoters were peripherally enriched, whereas transgenes with active developmentally regulated promoters were located in the nuclear interior. In contrast to low-copy transgenes, large repetitive gene arrays accumulated heterochromatic marks (H3K9me3 and H3K27me3) and were peripherally anchored in embryonic cells, correlating peripheral attachment with a heterochromatic state. Finally, we show that an intact nuclear envelope is required for efficient gene silencing because promoters located on transgene arrays are strongly up-regulated in animals depleted for the *C. elegans* lamin homolog LMN-1 or associated proteins.

GENERATION OF *lacO*-TAGGED LOW-COPY TRANSGENES TO MONITOR SUBNUCLEAR GENE POSITION DURING *C. ELEGANS* DEVELOPMENT

We have recently established the nematode *C. elegans* as a genetically tractable model system to investigate the function and mechanism of subnuclear chromatin organization (Meister et al. 2010a). We made use of this system to identify *cis*-acting elements that drive peripheral gene attachment. Using microparticle bombardment, we generated transgenes of developmentally regulated promoters driving a fluorescent reporter (*mCherry* or *his-24::mCherry*) in specified tissues that are flanked by arrays of *lacO* sites. The subnuclear position of these transgenes can therefore be tracked by expression of GFP (green fluorescent protein)-LacI, which accumulates at *lacO* arrays to form a fluorescent focus (Straight et al. 1996). Furthermore, the transgenes contain the neuronal gene *unc-119,* which was used as a selection marker (Fig. 1).

Microparticle bombardment results in chromosomally integrated transgenes with a copy number between 1 and 50 copies (Praitis et al. 2001; Meister et al. 2010a). The site of integration is random. This allows us to separate the localization potential of an individual promoter from the influence of surrounding genomic regions by analysis of several independent integration sites. Using this method, we generated multiple transgenes that harbor the muscle-specific promoter of the *myo-3* gene (2.5 kb upstream of ATG) or a fragment of the *pha-4* promoter that drives expression exclusively in the intestine (3.1 kb upstream of ATG). As a control, we included a strain that carries a *lacO*-tagged transgene with only the selection marker *unc-119*. We quantified the subnuclear distribution of these transgenes in embryonic cells as well as in three different differentiated tissues: muscle, intestine, and hypodermal and seam cells of ectodermal origin. Here, we focus our discussion on one of the *pha-4::his-24::mCherry* transgenes but emphasize that all of the transgenes that we generated behaved very similarly (Meister et al. 2010a).

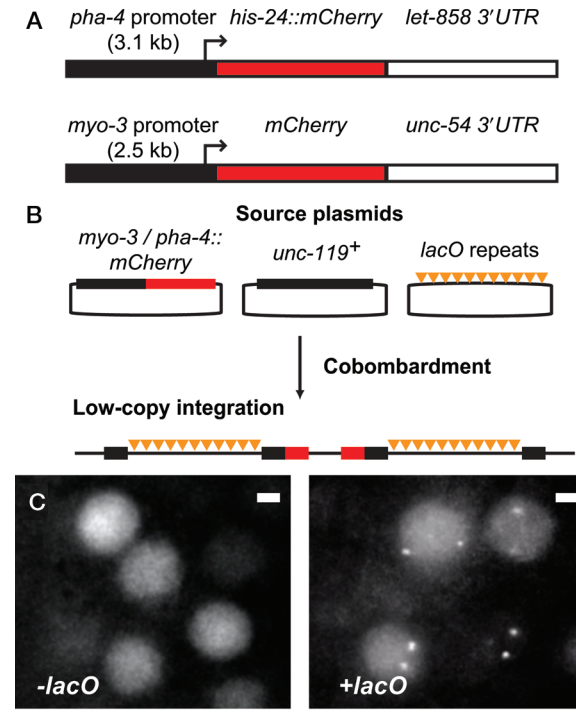

Figure 1. Visualization of *lacO*-tagged transgenes by GFP-LacI. (*A*) Developmental promoters used for generation of *lacO*-tagged transgenes. (*B*) Outline of transgenesis: Reporter transgenes of *pha-4* or *myo-3* promoter were cobombarded with the *unc-119* rescuing construct and repeats of 256 *lacO* sites. (*C*) *C. elegans* embryo expressing GFP-LacI only (*left*) or carrying in addition a *lacO*-tagged transgene insert (*right*) . Bar, 1 µm. (Adapted from Meister et al. 2010a.)

LOW-COPY TRANSGENES RANDOMLY DISTRIBUTED IN EARLY EMBRYOS

To determine the subnuclear position of the bombardment-derived *pha-4* transgene during early developmental stages, we labeled transgenic *C. elegans* embryos with antibodies directed against the nuclear lamina (LMN-1) to mark the nuclear periphery and with GFP to visualize the

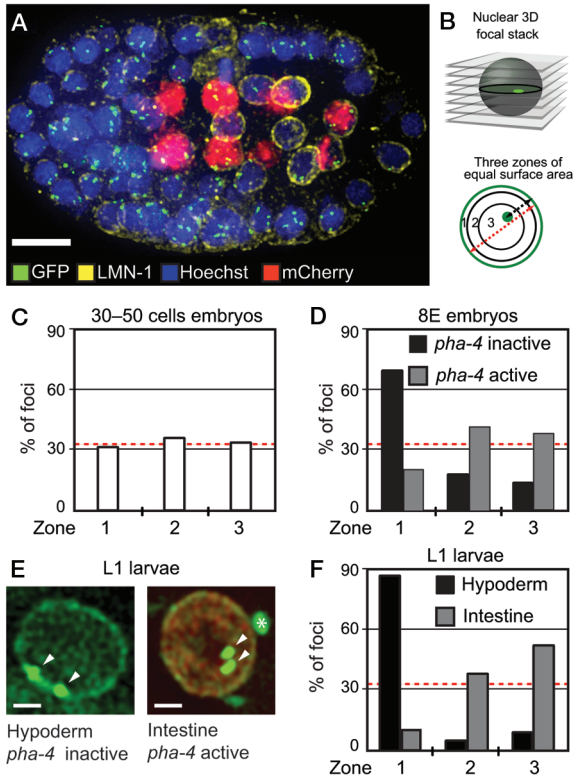

Figure 2. Differentiation-associated spatial separation of active and silent loci. (*A*) Maximum intensity projection of an 8E-stage *C. elegans* embryo carrying an integrated *lacO*-containing *pha-4::his-24-mCherry* transgene. The embryo is stained for LMN-1 (yellow), mCherry (red), GFP-LacI (green), and DNA (Hoechst blue). Bar, 5 µm. (*B*) Quantification of radial spot position. Nuclear cross section at the focal plane with highest spot intensity is divided into three concentric zones of equal surface, with zone 1 being the outermost. Spots are then binned into these three zones, and a randomly localized spot is equally distributed among the three zones. (*C*,*D*,*F*) Quantification of radial distribution of *pha-4* transgene at indicated developmental stages and cell types, using the method described in *B*. (Red line) Expected random distribution. (*E*) Hypodermal and intestinal nuclei of L1 larva carrying the same transgene as in *A*, crossed to a strain expressing GFP-LMN-1. Bar, 1 µm. (Arrowheads) GFP-LacI foci, (asterisk) autofluorescent gut granule. (Adapted from Meister et al. 2010a.)

position of the transgene. Intestinal precursor cells (E cells), where the *pha-4* promoter is active, could be identified by the presence of the mCherry signal (Fig. 2A). We subsequently acquired z stacks of embryos and for each GFP focus, we identified the focal plane with maximal signal. To quantify the radial distribution of transgene position, we divided the nuclear cross section at this focal plane into three concentric zones of equal surface and determined the relative occupancy of these three zones by the transgene (Fig. 2B). When repeated for many nuclei, the distribution of a randomly positioned locus yields equal occupancy of all three zones (33% of the foci in each zone). In contrast, a peripheral or a centrally located focus will be enriched in zone 1 or 3, respectively (Meister et al. 2010b).

We first quantified the subnuclear distribution of the *pha-4* transgene in very early embryos of 30–50 cells. At this developmental stage, most embryonic cells are still uncommitted to cell fate such that they can be differentiated into all classes of tissues by ectopic expression of the corresponding transcription factor (Sulston et al. 1983; Zhu et al. 1998; Yuzyuk et al. 2009). Analysis by three-zone scoring revealed that the *pha-4* transgene was randomly distributed throughout the nuclear space (Fig. 2C). This was true for independent transgene integrations of the *pha-4* promoter as well as for small arrays of the inactive tissue-specific *myo-3* promoter and the control strain carrying only the *unc-119*$^{+}$ selection marker (Meister et al. 2010a). Note that at this developmental stage, none of the transgene-borne promoters are active, and no mCherry signal was detected.

Previous work has shown inactive loci at the nuclear periphery in tissue culture cells and *Drosophila* embryos (Pickersgill et al. 2006; Guelen et al. 2008; Peric-Hupkes et al. 2010). Our results on the other hand suggest that transcriptional inactivity alone is insufficient for a perinuclear localization, at least in early *C. elegans* embryos.

LOW-COPY TRANSGENES OCCUPY DEFINED POSITIONS IN DIFFERENTIATED TISSUES

C. elegans embryos undergo cell-type commitment at a developmental stage where eight intestinal precursor cells are present (8E stage) (Zhu et al. 1998). This restriction in cell-fate potential coincides with a global compaction of chromatin (Yuzyuk et al. 2009).

In our system, the 8E stage can be easily identified by the presence of eight cells expressing HIS-24::mCherry (Fig. 2A, red). To investigate the effect of cell-type commitment and promoter activity on gene position, we next quantified the *pha-4* transgene position at the 8E stage in cells, where it is active in the eight intestinal precursors but silent in the rest of the embryo. In contrast to the situation in nuclei in the 30–50 cell embryos, the *pha-4* transgene was now enriched at the nuclear periphery in cells where the promoter was silent (Fig. 2D, black bars). On the other hand, in cells in which *his-24::mCherry* was expressed, the random distribution shifted slightly toward the nuclear center (Fig. 2D, gray bars).

Finally, we analyzed transgene distribution in fully differentiated cells of the first larval stage. Because immunofluorescence staining under conditions where nuclear structure is preserved is technically challenging in *C. elegans* larvae, we used a strain expressing GFP-tagged lamin (GFP-LMN-1) to mark the nuclear envelope, and we imaged live larvae. Three larval cell types were analyzed: ectodermal hypoderm and seam cells, which do not express *pha-4*, and intestine, where *pha-4* is active (Fig. 2E). Similar to the subnuclear distribution at the 8E stage, the transgene was enriched at the periphery in the hypodermis and seam cells, with nearly 90% of the foci located in zone 1. In contrast, in intestinal nuclei, the transgenes were depleted from the nuclear periphery and most often located in the nuclear center (Fig. 2F).

We performed equivalent experiments with a small transgene array (called *gwIs28*) carrying the muscle-spe-

cific *myo-3* promoter driving *mCherry*. Similar to the *pha-4* promoter, this transgene was randomly distributed in early embryonic nuclei. In contrast, the *myo-3* promoter-containing transgenes were enriched at the periphery in differentiated intestine and hypodermal nuclei. This is consistent with the promoter inactivity in these tissues. Importantly, we observed that the *myo-3* promoter transgene relocated to the nuclear center in muscle cells of L1 larvae, where this promoter is active (Meister et al. 2010a).

To summarize, these results indicate that in undifferentiated and uncommitted cells, developmentally regulated promoters have no specific subnuclear position. When cells undergo differentiation, genes are spatially separated based on their activity: Silent loci are relocated to the nuclear envelope, whereas active loci become enriched in the nuclear center. Similar to our findings, in a mouse embryonic stem cell (ESC) differentiation system, stronger lamin-DamID signals were found in differentiated astrocytes (ACs) than in pluripotent ESCs (Peric-Hupkes et al. 2010). However, in this case, it is unclear whether measurement of increased lamin interaction is due to technical differences in the analysis of ACs and ESCs or truly reflects a change in gene position. Moreover, it is unclear as to what degree pluripotent ESCs mimic early embryonic states.

COPY-NUMBER-DEPENDENT PERIPHERAL ATTACHMENT OF REPETITIVE TRANSGENES IN EMBRYONIC CELLS

Given that peripheral attachment of developmentally regulated promoters only occurs in differentiated tissues, we next asked what distinguishes embryonic nuclei from differentiated cells in their ability to recruit the same locus to the nuclear envelope. In principle, at least three explanations are possible: (1) Anchoring may be mediated by a binding factor recognizing the *pha-4/myo-3* promoter that is only expressed after differentiation in the cells where these are inactive. (2) The nuclear envelope in embryonic cells may be unable to bind silent chromatin and only gain this function by incorporation of additional factors during development. (3) Finally, perinuclear anchoring may be mediated by chromatin modifications that are deposited on developmentally regulated promoters after differentiation.

To test whether the nuclear envelope contains the proteins needed to bind heterochromatin in early embryonic cells, we next investigated a chromosomally integrated, *lacO*-containing *[pha-4::lacZ]* transgene that was generated by gonadal microinjection (Azzaria et al. 1996; Meister et al. 2010a). In contrast to transgenes generated by microparticle bombardment, gonadal injection results in large repetitive arrays of 300–500 copies of the injected DNA. Quantitative polymerase chain reaction (qPCR) revealed that the copy number of the *[pha-4::lacZ]* array used in this study was approximately 300 (Fig. 3A).

As a consequence of their repetitiveness, promoters on large arrays are often at least partially repressed and in a heterochromatic state (Hsieh and Fire 2000; Bessler et al. 2010). Therefore, visualization of the large *[pha-4::lacZ]* array by GFP-LacI allowed us to test whether a large heterochromatic domain was able to bind the nuclear envelope in early embryos. Indeed, these arrays were strongly enriched at the nuclear periphery with >80% of the foci found in the outermost zone (Fig. 3A,C). We similarly generated another chromosomally integrated, large array (called *gwIs4*) by microinjection and X-ray irradiation. This array contains a GFP-LacI expression plasmid, a plasmid with a *myo-3* promoter driving *rfp*, as well as *lacO* sites and can be visualized microscopically by GFP-LacI that is transcribed from the array itself. By qPCR, we estimate the plasmid copy number of *gwIs4* to be ~500 copies (Meister et al. 2010a). Quantification of the radial distribution of the *gwIs4* array revealed that, similar to the *[pha-4::lacZ]* array, this large array was strongly enriched at the nuclear envelope (Fig. 3E). Thus, the embryonic nuclear envelope is able to recruit large gene arrays.

To confirm that peripheral sequestration is size dependent and not reflective of the site of transgene integration, we isolated a strain in which the large *[pha-4::lacZ]* array had spontaneously reduced its copy number. qPCR confirmed that the array was about sixfold smaller than in the parental strain (Fig. 3B). Remarkably, this smaller array was no longer found almost exclusively at the nuclear envelope (Fig. 3D). Similarly, a bombardment-derived *myo-3::mCherry* transgene (*gwIs28*) with 10-fold fewer copies than the large *gwIs4[myo-3::rfp]* array was randomly distributed in early embryos (Fig. 3F) (Meister et al. 2010a).

We note that the low- and high-copy version of the *[pha-4::lacZ]* array are integrated at the same position on the chromosome because the low-copy array was generated from the high-copy array through a spontaneous recombination event. From this, we can therefore exclude that the differential subnuclear localization of these arrays is due to differences in their genomic integration site or method of transgenesis. We conclude that the high copy number of a transgene can direct it to the nuclear envelope. Furthermore, our findings argue against a sequence-specific DNA-binding factor initiating perinuclear gene attachment because the large and small *[pha-4::lacZ]* array share the same sequence composition but are differently localized in the nucleus. However, we cannot rule out that in later development, a tissue-specific factor also contributes to the anchoring event.

HIGH-COPY BUT NOT LOW-COPY TRANSGENES ACCUMULATE HETEROCHROMATIC MARKS

What could distinguish high- from low-copy transgenes in their nuclear localization? Given that promoters on large arrays have previously been shown to be subject to transcriptional silencing (Hsieh and Fire 2000) and to accumulate heterochromatic marks (Bessler et al. 2010), we tested whether small and large arrays differ in their histone modifications. Indeed, when we stained embryos that contain the large array *gwIs4[myo-3*::*rfp]* for the heterochromatic mark H3K9me3, we saw a spotty pattern with two bright spots in every nucleus. These bright foci colocalized precisely with the GFP signal marking the array position (Fig.

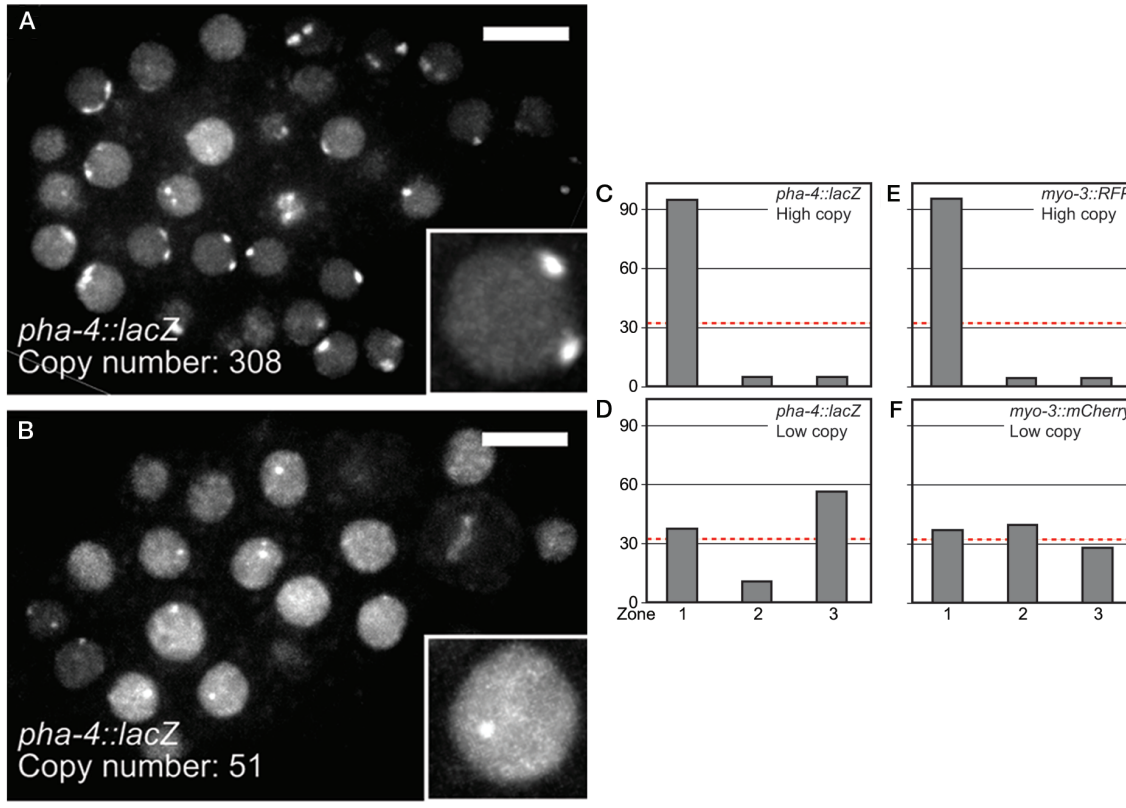

Figure 3. Peripheral anchoring of transgenes depends on their copy number. (*A*) Projection of six focal planes spanning 1.2 µm of an embryo carrying a large *[pha-4::lacZ]* array with 300 copies, visualized with GFP-LacI. Bar, 5 µm. (*B*) As in *A*, but the same transgene with spontaneously reduced size (51 copies). (*C–F*) Quantification of radial transgene positioning using three-zone scoring as described in Fig. 2B. (Red line) Random distribution. (*C*) Large *[pha-4::LacZ]* array shown in *A*. (*D*) Array with reduced size shown in *B*. (*E*) Large *gwIs4[myo-3::rfp]* array. (*F*) Bombardment-derived *gwIs28[myo-3::mCherry]* transgene with low copy number. (Adapted from Meister et al. 2010a.)

4A). Similarly, the *gwIs4* array was enriched for the Polycomb-associated mark H3K27me3 (Fig. 4B). In agreement with its repressed state, we found no H3K4me3, which marks active promoters, enriched on the array (Fig. 4C). This exclusion of H3K4me3 was not due to potential technical problems in the staining procedure because this modification was enriched on the *gwIs4* array in muscle cells of late-stage embryos when the *myo-3* promoter on the array is activated (Fig. 4D).

To compare the level of histone modifications on large and small arrays, we created a strain carrying both the large *gwIs4[myo-3::rfp]* array and the low-copy *gwIs28[myo-3::mCherry]* transgene. Hence, four GFP foci in every nucleus were detected. Two of these foci had an extended shape and correspond to the large array *gwIs4*, whereas the other two GFP signals had a spot-like appearance, with a size close to the diffraction limit, reflecting the smaller size of the low-copy transgene *gwIs28* (Fig. 3E,F; GFP). When we stained these embryos with antibodies against H3K9me3 and H3K27me3, only the two large GFP foci showed colocalization with either of the methylated histones (Fig. 3E,F). We therefore conclude that low-copy transgenes accumulate far less histone modifications typical for heterochromatic marks than large arrays.

The observation that copy-number-dependent perinuclear anchoring of repetitive transgenes correlated with their acquisition of H3K9me3 and H3K27me3 marks indicates that the heterochromatic state itself could serve as a signal for gene repositioning to the nuclear envelope. Genetic mutation of the histone methyltransferases that deposit these methyl marks will allow us to test this model.

C. ELEGANS LAMIN HOMOLOG LMN-1 REQUIRED FOR PERINUCLEAR ATTACHMENT OF GENE ARRAYS

To determine what might provide peripheral chromatin anchoring in *trans*, we tested whether an intact nuclear lamina was important for peripheral binding of gene arrays. *C. elegans* encodes a single lamin protein (LMN-1) that shares characteristics of both A- and B-type lamins. As previously described, down-regulation of *lmn-1* by RNA interference (RNAi) reduced LMN-1 levels in *C. elegans* embryos to <10% of wild-type levels (Fig. 5A) and caused arrest at early embryonic stages (Liu et al. 2000).

To test whether a functional nuclear lamina is required to maintain gene arrays at the nuclear envelope, L4 larvae carrying the large transgene array *gwIs4* were subjected to *lmn-1* RNAi for 24 h. Array position was determined in the 50-cell-stage embryonic progeny of these animals.

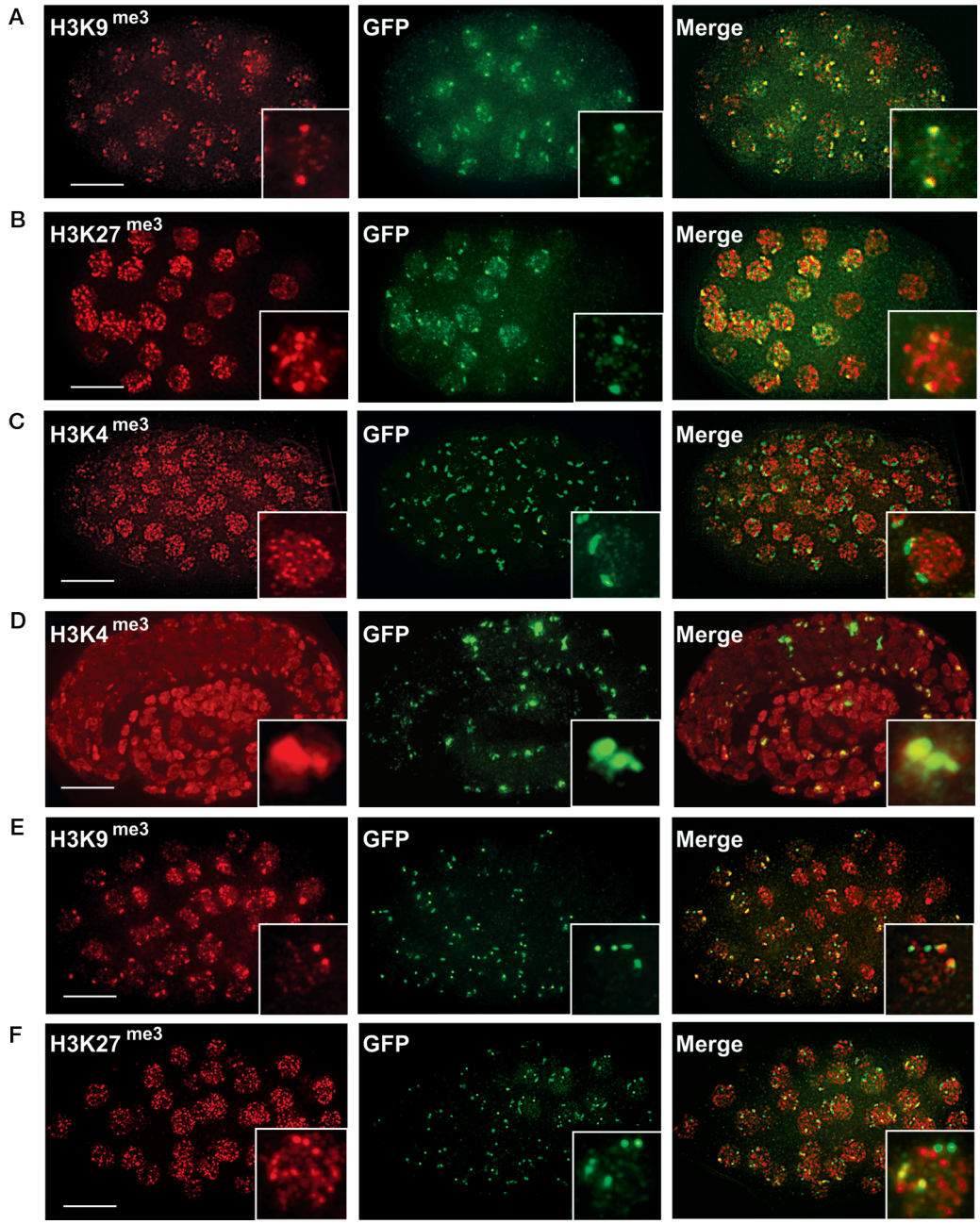

Figure 4. Large arrays but not small transgenes accumulate heterochromatic chromatin modifications. (*A–F*) Maximum intensity projections of *C. elegans* embryos carrying large *myo-3::rfp* array (*A–D*) or both large *myo-3::rfp* array and low-copy *myo-3::mCherry* transgene. (*E*,*F*) Embryos were stained with antibodies directed against indicated specific histone methylation marks and for GFP-LacI. Bars, 5 µm. (*A–C*,*E*,*F*) Early-stage embryos in which *myo-3* promoter is not active. (*D*) Late-stage embryo just before hatching. (*D*, inset) Muscle cell, where the *myo-3* promoter is active. (Adapted from Meister et al. 2010a.)

In 20% of the embryonic nuclei, at least one, and sometimes two, arrays shifted away from the nuclear envelope (Fig. 5B, *lmn-1[RNAi]*). In contrast, only 5% of the nuclei had at least one internal focus in embryos treated with a control RNAi vector (Fig. 5B,C; *mock[RNAi]*). An intact nuclear lamina is therefore necessary to retain arrays at the nuclear periphery. It remains unclear, however, whether there is a direct interaction between LMN-1 and the array or whether this involves lamin-associated chromatin-binding factors.

DEPLETION OF LMN-1 CAUSES STOCHASTIC DEREPRESSION OF ARRAY-BORNE PROMOTERS

The *gwIs4* array used in this study serves a dual purpose: The position of the GFP focus reflects the position of the transgene in the nucleus, and total GFP levels reflect the activity of the array-borne *baf-1* promoter that controls GFP-LacI expression. By monitoring GFP levels in animals carrying the genetic null allele *lmn-1(tm1502)*, we

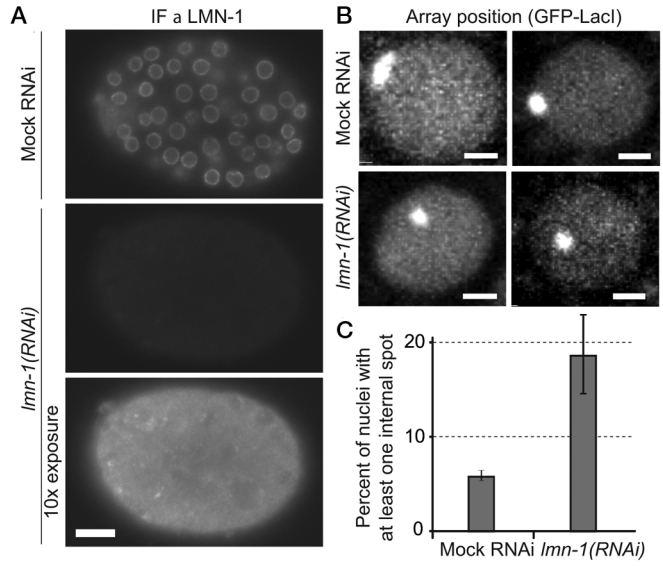

Figure 5. High levels of LMN-1 are required for efficient perinuclear array anchoring. (*A*) L4 larvae were subjected to *lmn-1* RNAi or mock RNAi, and embryonic progeny were immunostained for LMN-1 after 24 hours. Control and *lmn-1(RNAi)* embryos shown at same exposure and at 10-fold longer exposure for *lmn-1(RNAi)*. Note that LMN-1 is reduced to at least 10% of wild-type level but is still detectable. Bar, 5 µm. (*B*) GFP-LacI signal of nuclei of mock and *lmn-1(RNAi)*-treated embryos carrying large *myo-3::rfp* array. Shown is single focal plane of a stack. Bar, 1 µm. (*C*) Quantification of array detachment in 50-cell-stage embryos. Shown is fraction of nuclei that show at least one internal focus. Data reflect mean of two biologically independent replicas. Error bars indicate maxima and minima of data series.

could therefore test whether loss of array anchoring correlated with a reduced ability to maintain its partially repressed state.

In contrast to the high penetrance of embryonic death observed after RNAi against *lmn-1*, most animals homozygous for the genetic null allele *tm1502* complete embryogenesis and form sterile adults (Haithcock et al. 2005). This weaker phenotype of the *lmn-1* null allele is most likely due to rescue by the maternal load of *lmn-1* transcripts. Using the *tm1502* null allele, we examined whether the nuclear lamina had a role in array repression in differentiated tissues.

Whereas GFP-LacI was hardly detectable in adult wild-type animals (Fig. 6A), 67% of the *lmn-1(tm1502)* homozygous mutants had a few cells with very high GFP signal stemming from a derepressed *gwIs4* array (Fig. 6B). Array derepression seemed to occur stochastically in most tissues of the worm but always only in a subset of cells per animal. The reason for this low penetrance is unclear but may reflect either a stochastic loss of maternally con-

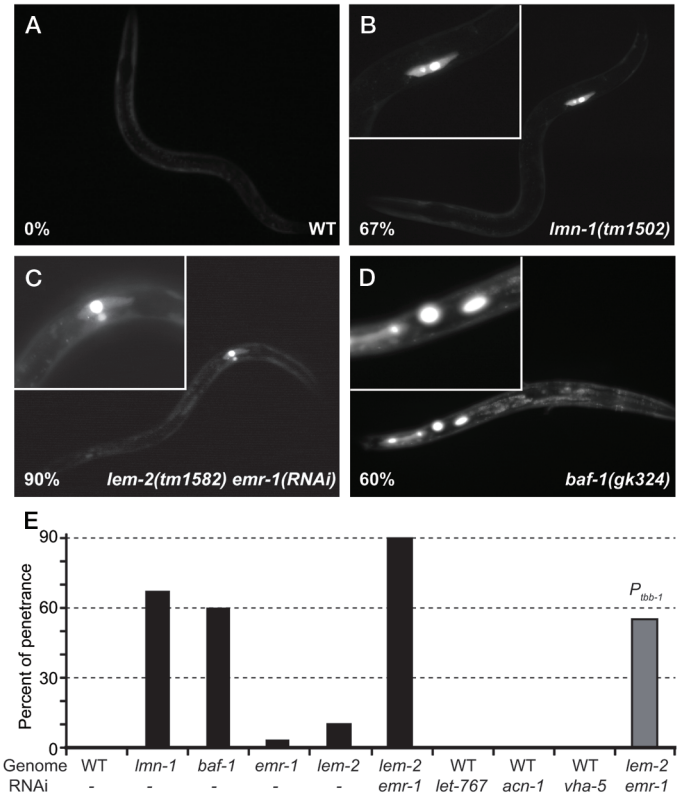

Figure 6. Intact nuclear envelope is required for efficient array repression. (*A–D*) GFP images of strains carrying the large integrated *gwIs4[baf-1:gfp-lacI; myo-3::rfp]* array in indicated backgrounds. GFP-LacI is barely detectable in wild type (WT) (*A*), but is found at high levels in *lmn-1(tm1502)* (*B*), *lem-2(tm1582)emr-1(RNAi)* (*C*), and *baf-1(gk324)* (*D*) mutants. Frequency of worms with at least one bright green nucleus indicated at bottom left of each image. (*E*) Quantification of the frequency of *gwIs4[baf-1::gfp-lacI; myo-3::rfp]* or *[tbb-1::mCherry-lacI]* (P_{tbb-1}) array derepression in indicated backgrounds and treated with indicated RNAi. Shown is fraction of worms with at least one bright nucleus.

tributed lamin during cell divisions or the stochastic nature of heterochromatin control over transcription. Nevertheless, this result suggests that the nuclear lamina is necessary to stably maintain transcriptional repression of arrays.

MUTATION OF LAMIN-INTERACTING FACTORS *BAF-1*, *EMR-1*, AND *LEM-2* PHENOCOPY ARRAY DEREPRESSION IN LMN-1–DEPLETED WORMS

LMN-1 interacts with the two transmembrane proteins, EMR-1 (homolog of human Emerin) and LEM-2 (hMAN1), that span the inner nuclear membrane (Liu et al. 2003). At their nucleoplasmic amino terminus, both proteins contain a LEM (LAP2, Emerin, and MAN1) domain that binds the small DNA cross-linking protein BAF-1 (Margalit et al. 2005).

Mutation of *baf-1*, or concurrent depletion of the lamin-associated LEM domain containing transmembrane proteins EMR-1 and LEM-2, causes phenotypes very similar to *lmn-1* mutation (Liu et al. 2000, 2003; Margalit et al. 2005), including chromosome segregation defects and promiscuous chromatin condensation. Therefore, we tested whether BAF-1 and EMR-1/LEM-2 were also required for array silencing. Indeed, we observed array derepression at high penetrance in animals homozygous for the *baf-1* null allele *gk324* (60%; Fig. 6D, *baf-1*). Single mutants of *emr-1* and *lem-2* had only minor defects in array silencing, in agreement with their previously described redundant functions (Fig. 6E, *emr-1* and *lem-2*) (Liu et al. 2003). By down-regulation of *emr-1* with RNAi in a *lem-2* null-mutant background, we depleted both proteins concurrently. This caused high-penetrance embryonic lethality (>98%; data not shown), as previously described for double RNAi against these two factors (Liu et al. 2003). The rare escapers arrested at the L2 or L3 larval stage, and 90% of these showed strong up-regulation of GFP-LacI in at least one cell (Fig. 6C, *lem-2, emr-1[RNAi]*).

Derepression cannot be explained exclusively by the larval arrest phenotype of *emr-1 lem-2(RNAi)* double-depleted animals because unrelated RNAi clones causing larval arrest (*let-767[RNAi]*, *acn-1[RNAi]*, and *vha-5[RNAi]*) did not cause array derepression (Fig. 6E). Finally, the observed increased levels of GFP-LacI are not due to a promoter-specific activation, because an *mCherry-LacI* transgene driven by the unrelated *tbb-1* promoter (β-tubulin) shows a similar derepression as the *baf-1* promoter (Fig. 6E, P_{tbb-1}). In conclusion, perturbation of the nuclear lamina by depletion of LMN-1, or its interacting partners BAF-1, EMR-1, and LEM-2, results in strong up-regulation of a usually silent transgene.

CONCLUSIONS

Our studies on nuclear organization in *C. elegans* have revealed that active and inactive developmentally regulated promoters are spatially separated in the nuclei of differentiated tissues of the first larval stage: Tissue-specific promoters are in the nuclear lumen when active and close to the nuclear periphery when silent. This is in agreement with studies in other experimental systems, where inactive genes were often found close to the nuclear lamina (Pickersgill et al. 2006; Guelen et al. 2008; Peric-Hupkes et al. 2010). In contrast to previous studies, we exogenously inserted fragments of developmentally regulated promoters (3.1 kb and 2.5 kb for *pha-4* and *myo-3*, respectively) at random sites in the genome. We analyzed multiple insertions of these two different promoters in a range of tissues (intestine, muscle, hypdodermal cells, and seam cells). In all cases, the developmentally regulated promoters assumed a position within the nucleus that reflected their activity state, i.e., inactive promoters were perinuclear and active promoters internal. Our results therefore strongly suggest that the tissue-specific promoter fragments that we inserted are sufficient to control subnuclear position.

Although low-copy transgenes were randomly distributed in early embryonic cells, the nuclear envelope is capable of recruiting chromatin even at this early developmental stage. Much larger arrays of transgenes (repetitive copy number 250–500) were strongly enriched at the nuclear periphery in embryos as well as larval cells. This peripheral sequestration correlated with an accumulation of heterochromatic marks and is unlikely to be dictated by the site of integration or by sequence-specific binding factors. Promoters of identical sequence inserted at the same locus, in reduced copy number, were not peripherally enriched. It is therefore tempting to speculate that the formation of heterochromatin itself drives nuclear organization. According to such a model, the relocation of inactive low-copy transgenes to the nuclear envelope during development may be a consequence of a change in chromatin state of the promoters upon cell commitment and differentiation. The exact nature of the molecular players involved in this process remains to be identified.

Although our findings suggest that deposition of heterochromatic marks itself contributes to peripheral chromatin anchoring, we find that a functional nuclear envelope is required, for both chromatin sequestration and efficient silencing. Arrays are delocalized upon RNAi against *lmn-1*, whereas depletion of the nuclear envelope components LMN-1 and BAF-1 or codepletion of EMR-1 and LEM-2 caused a stochastic derepression of heterochromatic transgene arrays. This result is reminiscent of a recent study showing up-regulation of a normally silent testis-specific gene cluster in flies deficient for the *Drosophila* lamin homolog lamDm0 (Shevelyov et al. 2009).

To summarize, our data suggest that the peripheral sequestration of heterochromatin reinforces its silent state (Fig. 7). This may occur through local abundance of histone methyltransferases and methylhistone-binding factors or through indirect effects such as late replication or local depletion of active RNA polymerase. The value of RNAi screens in resolving this is an obvious advantage of the *C. elegans* system described here.

MATERIALS AND METHODS

Molecular Biology and Transgenic Strains

Table 1 lists the strains used in this study; most strains and plasmids are described elsewhere (Meister et al. 2010a).

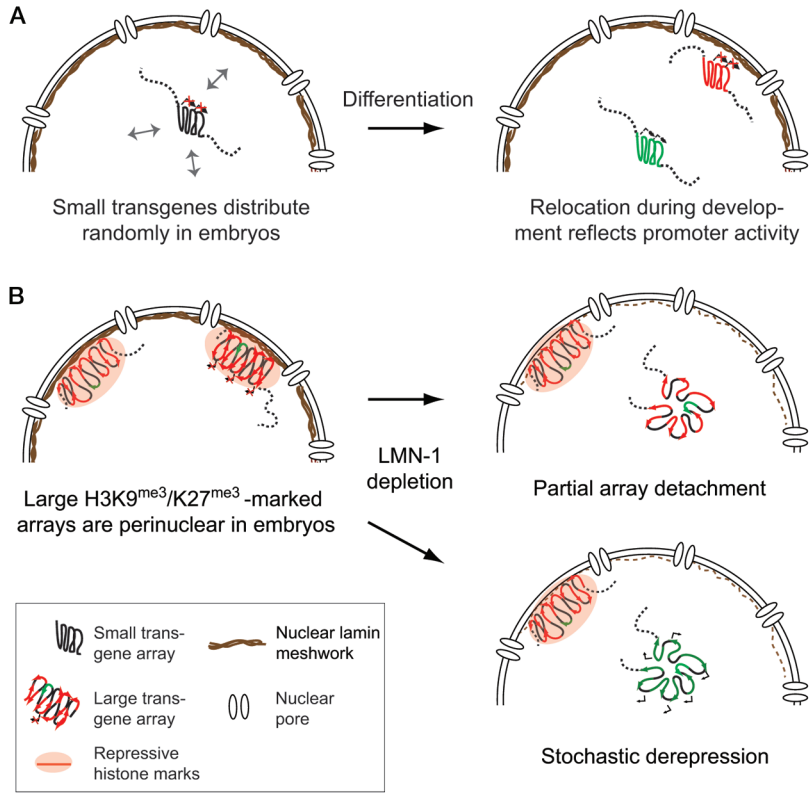

Figure 7. Model for function of perinuclear gene attachment in gene silencing. (*A*) Low-copy transgenes are randomly distributed in undifferentiated embryonic cells, independent of promoter activity. During differentiation, inactive promoters relocate to nuclear envelope, and active promoters become enriched in nuclear center. (*B*) Large heterochromatic transgene arrays are bound to nuclear lamina in wild-type embryos, and promoters on the transgene are transcriptionally repressed. Depletion of LMN-1 by RNAi or its mutation causes release of large arrays from the nuclear lamina. Not all arrays detach from the nuclear periphery; some LMN-1 remains due to incomplete RNAi or because of remaining maternally contributed LMN1 protein. Detachment of arrays impairs their efficient silencing, such that a fraction of the arrays gets derepressed stochastically.

Table 1. Strains used in this study

Strain name	Genotype	References
N2	*Wild-type Bristol isolate*	
GW76	*gwIs4[myo-3::rfp baf-1::gfp-lacI let-858 3′UTR]*	Meister et al. (2010a)
GW115	*gwIs4; lmn-1(tm1502)/hT2[bli-4(e937) let-?(q782) qIs48]*	This study
GW164	*gwIs4; emr-1(gk119)*	This study
GW201	*gwIs4; lem-2(tm1582)*	This study
GW205	*gwIs4; baf-1(gk324)/hT2[bli-4(e937) let(q782) qIs48]*	This study
GW318	*gwIs4; gwIs28[myo-3::mCherry; 256xlacO; unc-119^{+}]; unc-119(ed3)*	Meister et al. (2010a)
GW395	*gwIs39[baf-1::gfp-lacI::let-858 3′UTR; vit-5::gfp]; unc-119(ed3)*	Meister et al. (2010a)
GW397	*gwIs39; gwIs28; unc-119(ed3)*	Meister et al. (2010a)
GW429	*gwIs39; gwIs59[pha-4::mCherry::his-24; 256xlacO; unc-119^{+}]; unc-119(ed3)*	Meister et al. (2010a)
GW430	*gwIs25 [tbb-1::wmCherry-LacI::tbb-2 unc-119(+)]; unc-119(ed3); lem-2(tm1582)*	This study
GW431	*gwIs39; gwIs59; ygIs[lmn-1::lmn-1::gfp::lmn-1 3′UTR, unc-119^{+}]*	Meister et al. (2010a)
GW457	*gwIs4; gwIs39*	This study
GW470	*gwIs39; caIs*[pha-4::lacZ; rol-6(su1006)]; unc-119(ed3)*	This study
GW471	*gwIs39; caIs[pha-4::lacZ; rol-6(su1006)]; unc-119(ed3)*	Meister et al. (2010a)

*Transgene with reduced copy number.

Strains were made by back-crossing deletion alleles obtained from the *C. elegans* knockout consortium to wild-type N2 animals and subsequently to GW76. GW76 is an 8x outcrossed strain that carries a large integrated array expressing GFP-LacI under control of a *baf-1* promoter (*gwIs4*) but is otherwise wild type. The following alleles were used: *lmn-1(tm1502)*, *emr-1(gk119)*, *lem-2(tm1582)*, and *baf-1(gk324)*. For *lmn-1* RNAi experiments, GW76 was supplemented with a transgene (*gwIs39*) expressing GFP-LacI under the *baf-1* promoter to enhance the GFP signal for microscopy. *gwIs39* does not contain *lacO* sites and is therefore not visible as a fluorescent focus.

RNAi and Microscopy

RNAi was performed by feeding according to standard methods (Timmons et al. 2001). RNAi clones were obtained from the Vidal library (Rual et al. 2004) (*lmn-1*) or Ahringer library (Kamath et al. 2003) (all others). The empty vector L4440 was modified by removal of an EcoRV fragment containing a 25-bp stretch of perfect identity to linker DNA in the *gfp-lacI* construct and was used as control RNAi. For *lmn-1* RNAi, L4 larvae were subjected to RNAi, and embryonic progeny were analyzed after 24 h. To this end, embryos were mounted on 2% agarose pads, and 3D focal stacks were acquired on a spinning-disk confocal microscope as described by Meister et al. (2010a). Stacks of images were quantified manually using ImageJ. For derepression assays, L1 larvae were subjected to RNAi, and progeny was imaged with a widefield Axioplan microscope using a 20x objective (Zeiss). Immunofluorescence and quantification of radial spot position were performed as in Meister et al. (2010a). A Gaussian filter was applied to the mCherry channel in Fig. 2A (0.3-µm radius) and to the GFP signal in Fig. 3A,B (0.05-µm radius).

Copy-Number Quantification

Genomic DNA was isolated according to standard methods. Copy number was determined by qPCR from the ratio of amplicons from the ampicillin-resistance marker on the plasmid backbone and the single-copy locus *lmn-1* as described in Meister et al. (2010a).

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Repetitive Transgenes in *C. elegans* Accumulate Heterochromatic Marks and Are Sequestered at the Nuclear Envelope in a Copy-Number- and Lamin-Dependent Manner

B.D. Towbin, P. Meister, B.L. Pike, et al.

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