

Gene positioning and genome function

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Abstract The eukaryotic genome is packaged as chromatin within the three-dimensional nuclear space. Decades of cytological studies have revealed that chromosomes and genes are non-randomly localized within the nucleus and such organizations have important roles on genome function. However, several fundamental questions remain to be resolved. For example, what is required for the preferential localization of a gene to a nuclear landmark? What is the mechanism underlying gene repositioning in the nucleus? How does subnuclear gene positioning regulate gene transcription? Recent studies have revealed that several factors such as DNA sequence composition, specific regulatory sequences, epigenetic modifications, chromatin remodelers, post-transcriptional regulators and nuclear architectural proteins can influence chromatin dynamics and gene positioning in a gene-specific manner among organisms from yeast to human. In this review, we discuss some recent findings as well as experimental tools to investigate subnuclear gene positioning and to explore its implications in genome functions.

Keywords nucleus, transcription, gene positioning, epigenetics, nuclear lamina, chromatin

Introduction

Genetic information—from the whole chromosome level down to the individual gene level—is arranged in a non-random manner within the cell nucleus (Misteli, 2007). Heterochromatin is preferentially found at the nuclear periphery or around nucleoli (Haaf and Schmid, 1991). Gene-rich or smaller chromosomes tend to be located at the nuclear interior, whereas gene-poor or larger chromosomes prefer to be present at the nuclear periphery (Croft et al., 1999; Sun et al., 2000; Boyle et al., 2001; Parada and Misteli, 2002; Tanabe et al., 2002). Remarkably, many of these arrangements are evolutionarily conserved (Tanabe et al., 2002; Solovei et al., 2009). Subnuclear localizations of genetic elements also correlate with DNA sequence compositions and replication timing. GC-rich portions of the genome are more internally localized than AT-rich portions (Heppenger et al., 2008). Late-replicating genomic regions are generally located at the nuclear periphery, and early-replicating regions preferentially reside in the nuclear interior (Dimitrova and Gilbert, 1999; Berezney et al., 2000). Some

gene loci occupy non-random positions with respect to chromosome territories, to specific genes, or to heterochromatin in a tissue or cell-specific manner (Gilbert, 2001; Takizawa et al., 2008b; Geyer et al., 2011). Furthermore, repositioning of gene loci often occurs during differentiation (Kosak et al., 2002; Ragozy et al., 2006; Misteli, 2007; Yao et al., 2011) and under pathological conditions such as dilated cardiomyopathy (Mewborn et al., 2010) and breast cancer (Meaburn and Misteli, 2008; Meaburn et al., 2009).

A large body of cytological observations demonstrates that the spatial arrangement of chromatin within the three dimensional (3D) structure of the nucleus may have functional implications on genome functions. However, we are just beginning to understand its relevance to related physiological processes. Here we provide an overview of recent results and techniques developed toward understanding the mechanisms of gene positioning and discuss its impact on genome functions, with an emphasis on transcriptional regulation.

Non-random gene positioning in the nucleus

The nucleus is divided into distinct chromosomal and protein-rich compartments, which include the centromere clusters, nuclear lamina (NL), nuclear pore complexes (NPCs),

Received March 10, 2014; accepted April 28, 2014

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interchromatin granules, promyelocytic leukemia (PML) bodies, Cajal bodies, nuclear speckles and nucleoli (Spector, 2001; Pederson, 2002; Lamond and Sleeman, 2003). The spatial position of genes to these subnuclear compartments can influence gene transcription and other DNA-dependent processes (Isogai and Tjian, 2003; Fraser and Bickmore, 2007; Misteli, 2007; Takizawa et al., 2008b; Ferrai et al., 2010) (Fig. 1). For example, transcriptionally silent *CD4* and *CD8* genes were selectively associated with Ikaros centromere clusters in B cells (Brown et al., 1997). In hematopoietic progenitors and pro-T cells, inactive *IgH* and *Igk* loci were preferentially positioned at the nuclear periphery. However, in pro-B cells these loci were repositioned away from the nuclear periphery and underwent large-scale compaction (Kosak et al., 2002). In CD4 CD8 double-negative thymocytes where T cell receptor β (*Tcrb*) alleles were shown to recombine asynchronously, alleles associated with the nuclear lamina were suppressed for V(D)J recombination (Chan et al., 2013). Non-random gene positioning was observed for genes with mono-allelic expression. In cortical astrocytes, two alleles of the *GFAP* gene occupied differential radial positions, with the active allele located toward the nuclear interior (Takizawa et al., 2008a).

Repositioning of a gene toward heterochromatin or the nuclear periphery is generally associated with gene repression. For example, in resting murine B lymphocytes, $\lambda 5$, *CD8 α* , and *Rag* gene loci were not associated with centromeric γ -satellite regions; upon mitogen induced gene repression, these loci were associated with centromeres (Brown et al., 1999). In developing T cells, *Rag* and *TdT* loci were repositioned to centromeric domains upon differentiation-induced heritable silencing of these genes (Brown et al., 1999). During T helper cell (Th1) differentiation, Th2 cytokine regulators *GATA-3* and *c-maf* loci were repressed and repositioned to centromeric heterochromatin and/or the nuclear periphery (Hewitt et al., 2004). During neural

differentiation, downregulation of *Fgf8* expression was correlated with a more peripheral position of this locus (Patel et al., 2013). Similarly, repression of the *hunchback* gene in *Drosophila* neuroblasts, which led to the loss of neural progenitor competence, was correlated with its repositioning to the nuclear periphery (Kohwi et al., 2013). These correlative observations indicate that the nuclear periphery is generally an inactive subnuclear compartment for gene transcription. Furthermore, artificial tethering of endogenous genes and transgenes to the NL supports the repressive roles of the nuclear periphery on gene transcription (Andrulis et al., 1998; Finlan et al., 2008; Kumaran and Spector, 2008; Reddy et al., 2008).

Repositioning of genes away from the nuclear periphery often leads to gene activation or increased transcription levels. For example, during neural differentiation, *Pax6* and *Irx3* gene loci underwent both decompaction and displacement toward the nuclear interior concurrent with their transcriptional activation (Patel et al., 2013). During adipocyte differentiation, the adipogenesis genes *PPARG*, *FABP4*, *GATA2*, *SREBF1*, *CEBPB* and *CREB* also repositioned to the nuclear interior with increased transcriptional activities (Szczerbal et al., 2009). Interestingly, the *GATA2* gene which was expressed only in the initial stage of adipocyte differentiation, returned to the nuclear periphery during later stages of differentiation (Szczerbal et al., 2009). In mouse mammary epithelial cells, the casein gene cluster (*CSN*) was located at the nuclear periphery (Ballester et al., 2008; Kress et al., 2011). Upon stimulation by lactogenic hormones, the *CSN* locus repositioned away from the nuclear periphery and outside its chromosome territory (Ballester et al., 2008; Kress et al., 2011). In embryonic stem (ES) cells, the proneural regulator gene *Mash1* was transcriptionally inactive and was preferentially associated with the nuclear periphery. After retinoic acid-induced neural differentiation, *Mash1* was expressed and the locus repositioned away from the nuclear

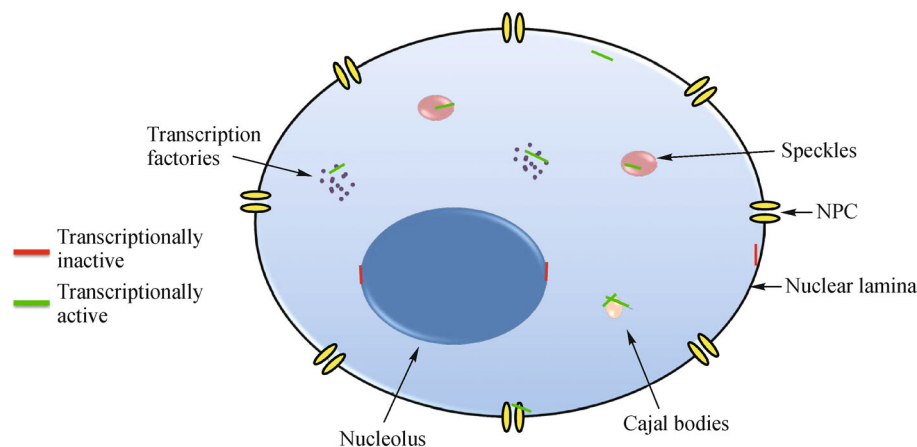


Figure 1 Gene positioning and transcriptional status. The cartoon depicts positioning of transgenes or endogenous genes to different subnuclear compartments and their transcriptional status. Genes or transgenes associated with NPCs, Cajal bodies and transcriptional factories are generally active; genes or transgenes associated with the NL or nucleoli are often inactive.

periphery (Williams et al., 2006). This gene repositioning appeared to be selective for cells from neural-committed lineages, as this repositioning was not observed in ES-derived mesodermal cells, keratinocytes or T cells (Williams et al., 2006). The pluripotency genes *Sox2*, *Oct4* and *Nanog* exhibited random distribution in mouse embryonic fibroblasts (MEFs) (Jost et al., 2011). In induced pluripotent stem (iPS) and ES cells where these genes are active, these loci were positioned away from the nuclear periphery (Jost et al., 2011). Interestingly, some genes can be active when located at the nuclear periphery. For example, *MyoD1* in mouse myoblasts (Lee et al., 2006; Yao et al., 2011) and β -globin in mouse erythroid cells (Ragoczy et al., 2006) were transcriptionally active at the nuclear periphery. After differentiation, these genes repositioned to the nuclear interior with a concomitant increase in transcription.

Repositioning of genes to NPCs was observed to correlate with changes in gene transcription. The NPCs were shown to create transcriptionally active microenvironments by inhibiting the spreading of heterochromatin (Ishii et al., 2002). In fact, numerous genes including *INO1*, *HXK1*, *GAL1*, *GAL2*, *GAL7*, *GAL10*, *SUC2*, *HSP104* and mating pheromone inducible genes repositioned close to NPCs upon transcriptional induction in yeast (Brickner and Walter, 2004; Casolari et al., 2004; Casolari et al., 2005; Cabal et al., 2006; Dieppois et al., 2006; Schmid et al., 2006; Taddei et al., 2006; Sarma et al., 2007). Interactions with NPCs were shown to constrain the mobility of these loci to a two-dimensional sliding motion along the nuclear envelope (Cabal et al., 2006). Similarly, correlation of active transcription and recruitment to NPCs was observed in mammals. In murine erythroid cells, the β -globin locus present at the nuclear periphery during early transcriptional activation was colocalized with NPC components (Ragoczy et al., 2006). These observations suggest that gene recruitment to the nuclear pore can play a positive role in gene transcription both in yeast and mammals.

Gene positioning to other nuclear domains upon changes in transcription was also reported. In erythroid cells, the actively transcribed globin genes *Hbb* and *Hba* were colocalized with several other active genes in shared transcription factories (Osborne et al., 2004; Schoenfelder et al., 2010) or nuclear speckles (Brown et al., 2008b). In human fibroblasts, *Collagen Ia1* and β -actin genes showed a preferential association with nuclear speckles, which are nuclear domains enriched with splicing factors and poly(A) RNA (Xing et al., 1995). Similarly, muscle specific genes *cMyHC* and *Myogenin* were colocalized with nuclear speckles upon myogenic differentiation (Moen et al., 2004). The transcriptionally active cytomegalovirus immediate early gene cluster was also associated with nuclear speckles (Dirks et al., 1997). Upon induction, U2 snRNA arrays were associated with Cajal bodies, which play roles in the biogenesis of spliceosomal small ribonucleoprotein particles (snRNPs) (Dundr et al., 2007).

The correlations between gene positioning and its

transcriptional activities appeared to be gene-specific. The transcriptional status of certain genes was reported to be independent of their positions relative to the nuclear periphery. For example, the neighboring 2 Mb region around the *Mash1* gene containing inactive non-neural genes *Pah* and *Igf1* moved away from the nuclear periphery upon neural differentiation (Williams et al., 2006). Similarly, the *IFN- γ* locus was constitutively associated with the nuclear periphery, irrespective of cell lineages or when primed for expression in Th1 cells (Hewitt et al., 2004).

Approaches to visualize gene loci in cells

Visualizing gene loci, chromatin domains and chromosome conformation within the cell is essential to understand the connections between the spatiotemporal organization of the genome and its functions. Imaging gene positioning has been performed both in fixed cells using fluorescence *in situ* hybridization (FISH) (Fig. 2A) and in live cells with the *lac* operator/*lac* repressor (*lacO/lacI*) system (Fig. 2B) (Belmont et al., 1999). These two approaches complement each other. 3D FISH allows detecting multiple genomic segments in the same nucleus, and the *lacO/lacI* system allows observing large-scale chromatin dynamics in living cells. In combination with immunofluorescence staining, relative subnuclear positioning of genes with respect to different nuclear landmarks can be analyzed.

DNA FISH (Levsky and Singer, 2003) on two and three-dimensionally preserved nuclei is a major tool to study subnuclear positioning of genes and chromosome territories. Briefly, adherent cells were grown on cover glasses or chamber slides, fixed with formaldehyde, denatured and hybridized with digoxigenin (DIG) or Biotin-labeled DNA probes. After incubation with fluorescently-labeled antibodies, FISH signals could be visualized under a fluorescent microscope (Fig. 2A). Multicolor DNA FISH, in which distinct genomic regions can be labeled with different epitopes/fluorescent probes in the same cell, was carried out in some cells, such as in erythroblasts and lymphoblast nuclei (Schröck et al., 1996; Brown et al., 2006; Brown et al., 2008b). The resolution of DNA FISH with light microscopy is around 200–250 nm. At the light microscopy level, the 3D-FISH procedure did not lead to obvious alterations in general nuclear distributions of chromatin (Solovei et al., 2002). However, electron microscopy showed that 3D-FISH can cause substantial changes in the ultrastructure of chromatin (Solovei et al., 2002). This inherent technical limitation of 3D-FISH suggests that it is not the preferred approach to study nuclear ultrastructures at the nanometer scale (Solovei et al., 2002). Cryo-FISH offers improved preservation of the nuclear ultrastructure and increased spatial resolution in the z-axis by preparing ultrathin cryosections (Branco and Pombo, 2006) and was used to identify interacting DNA loci in cells (Simonis et al., 2006).

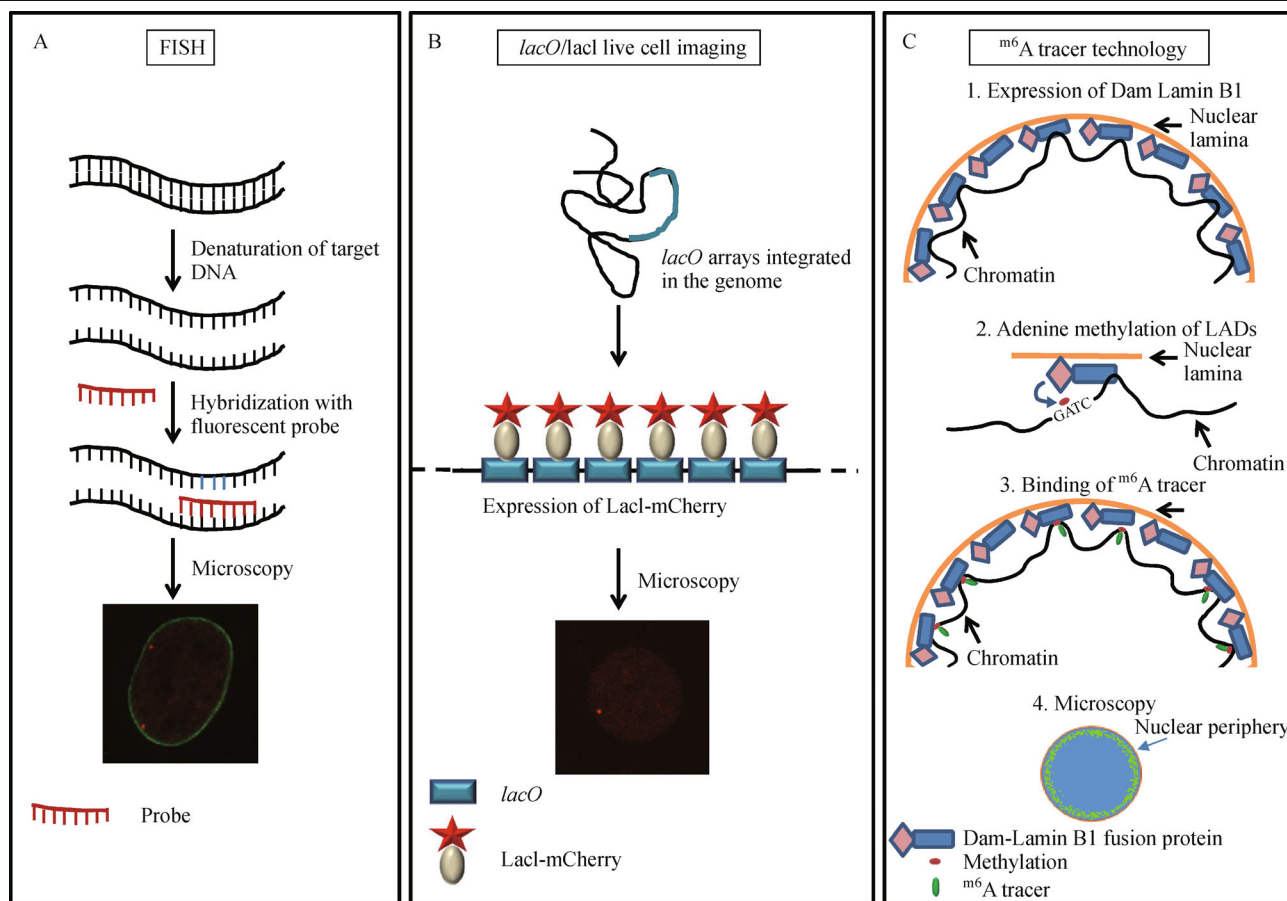


Figure 2 Imaging tools to detect gene positioning. (A) DNA FISH. Adherent cells grown in cover glasses or chamber slides were fixed with formaldehyde, denatured and hybridized with digoxigenin (DIG) or Biotin-labeled DNA probes (red). After incubation with fluorescently-labeled antibodies, FISH signals could be visualized under a fluorescent microscope. (B) *lacO*/lacI system. The *lacO* repeats were stably inserted adjacent to a gene of interest. The fluorescently-tagged *lacO* binding lac repressor (red) was expressed in these cells. Binding of lacI repressor to *lacO* repeats enabled the visualization of these loci in live cells. (C) ^{m6A} tracer technology. The *E. coli* adenine methyltransferase Dam enzyme was fused with Lamin B1 and expressed in cells. The genomic regions in molecular contact with Lamin B1 were tagged *in vivo* with adenine-6-methylation (^{m6A}) (red). When ^{m6A}-Tracer — a fluorescently-tagged DpnI truncation mutant that binds to G^{m6A}ATC (green) — was expressed in cells, the LADs could be visualized and tracked.

An alternative to DNA FISH is to use high-affinity protein-DNA interactions to visualize specific gene loci and to image their dynamics in live cells. The most widely-used system for imaging gene loci *in vivo* is the *lacO*/lacI system (Robinett et al., 1996). Briefly, tens to hundreds of *lac* operator (*lacO*) repeats (the 17 bp consensus sequence recognized by the lac repressor) were inserted adjacent to a gene of interest (Fig. 2B). The lac repressor lacI fused to a fluorescent protein with a nuclear localization signal was then expressed in these cells. The fluorescently-tagged lacI binds to *lacO* repeats and enables the visualization of these loci in live cells (Robinett et al., 1996). Similar systems using other bacterial DNA binding proteins (*tetO*/tet repressor) were also used to probe chromatin dynamics in live cells (Matzke et al., 2005). To visualize chromatin dynamics in *Caenorhabditis elegans*, *lacO* repeats were inserted into the genome either as high-copy number gene arrays (Towbin et al., 2010), or as low-copy number transgenes (Meister et al., 2010; Towbin et al., 2010).

DNA recombination approaches (O’Gorman et al., 1991) and genome editing methods such as ZFN (zinc-finger nucleases), TALEN (transcription activator-like effector nuclease) and CRISPR/cas9 (clustered regulatory interspaced short palindromic repeats)-based techniques (Gaj et al., 2013) can be used to generate *lacO* insertions at specific gene loci. Recently, imaging of endogenous genomic loci was performed in living mammalian cells using TALEN (Miyanari et al., 2013) and CRISPR-based technology (Chen et al., 2013). The CRISPR system includes a Cas9 protein that recognizes DNA sequences, with its target specificity solely determined by a small guide (sg) RNA and a protospacer adjacent motif (PAM) (Jinek et al., 2012). EGFP-tagged endonuclease-deactivated dCas9 protein and a structurally optimized sgRNA that binds to target DNA were used to visualize the spatial organization and dynamics of repetitive and non-repetitive genomic sequences (Chen et al., 2013). The CRISPR imaging method may perturb gene expression, which can be minimized by targeting the distal downstream

region of the gene or upstream region of the promoter (Chen et al., 2013).

Lamin associated chromatin domains (LADs) can be imaged in single cells using a “molecular contact memory” approach called ^{m6}A-Tracer technology (Fig. 2C) (Kind et al., 2013). In this method, the *E. coli* DNA adenine methyltransferase (Dam) enzyme was fused with chromatin interacting proteins such as Lamin B1 (van Steensel and Henikoff, 2000). The genomic regions in molecular contact with Lamin B1 were tagged *in vivo* with adenine-6-methylation (^{m6}A), a modification not found in higher eukaryotes. When ^{m6}A-Tracer—an EGFP-tagged DpnI truncation mutant that binds to G^{m6}ATC—was expressed in these cells, LADs could be visualized and tracked in single human cells. This study showed that only ~30% of LADs were still positioned at the nuclear periphery after mitosis and a substantial portion of LADs were stochastically reshuffled in daughter cells (Kind et al., 2013).

Factors required for gene positioning

Despite substantial efforts in detecting non-random gene positioning in cells, it remains unclear how the position of a particular gene is established in the nuclear space and what regulatory factors are involved. Recent studies suggest that several factors including genetic elements, histone modifications, transcription activators and chromatin remodelers may play a role or act in parallel to modulate the subnuclear localizations of genes.

The ease of genetic manipulation in yeast has enabled identifying specific elements and factors required for gene positioning to the nuclear periphery. A number of regulatory DNA sequences required for gene positioning were identified in yeast. For example, the recruitment of *GAL* locus (*GAL1/7/10*) to the NPC required the UASg element and the TATA-box (Schmid et al., 2006). DNA zip code sequences (gene recruitment sequences I and II) in the *INO1* gene promoter were required for its recruitment to the NPC and full transcriptional activation after inositol starvation (Brickner and Walter, 2004; Brickner et al., 2007; Ahmed et al., 2010). These DNA zip codes were sufficient to target an ectopic locus to the nuclear periphery even in the highly divergent fission yeast *S. pombe*, suggesting that this targeting mechanism is evolutionarily conserved (Ahmed et al., 2010). Additionally, recruitment of the *GAL* genes to the nuclear pore was mediated by the transcription coactivator complex SAGA subunits Sus1 and Ada2 (Cabal et al., 2006; Luthra et al., 2007; Green et al., 2012), Gal4 activator (Schmid et al., 2006), Glucose repressor Mig1 (Sarma et al., 2007), and some NPC components (Cabal et al., 2006; Taddei, 2007).

One prominent model to explain gene positioning to the nuclear periphery/NPC is the gene gating model (Blobel, 1985), which suggests that active genes are located near the

NPC to ensure rapid gene expression and to facilitate efficient export of mRNAs from the nucleus (Blobel, 1985; Cabal et al., 2006). In congruence with the model, post-transcriptional regulation was found to be associated with gene positioning to the NPC. Association of α -factor-induced genes with NPC component Mlip1 was shown to be sensitive to RNase treatment (Casolari et al., 2005). Moreover, upon switching the 3' untranslated region (UTR) of *GAL* transgenes from GALpA to TDH₃pA, the transgene occupied an intermediate location that was neither peripheral nor central compared to *GAL* transgenes under galactose or glucose culture conditions, respectively (Abruzzi et al., 2006). Similarly, deleting the 3'UTR of the *HXX1* gene abolished its localization to the nuclear periphery (Taddei et al., 2006). mRNPs and mRNA export factors were also implicated in tethering genes to the NPC in yeast (Abruzzi et al., 2006; Cabal et al., 2006; Drubin et al., 2006; Vodala et al., 2008). Interestingly, mRNA processing and/or export of *GAL1* mRNA was not delayed in *ada2* Δ mutant cells, where *GAL1* loci were no longer tethered to the NPC (Green et al., 2012). Instead, localization to the nuclear periphery dampened the initial *GAL* gene induction and was required for rapid gene repression after inactivation by glucose (Green et al., 2012). Therefore, NPC-associated *GAL1* loci may provide yeast cells with a mechanism to quickly respond to changing environmental conditions (Green et al., 2012).

In metazoans, the factors required for gene positioning are less well defined. Nuclear lamina-associated regions are preferentially localized at the nuclear periphery and have been extensively characterized both at the DNA sequence and the epigenome level (Guelen et al., 2008; Peric-Hupkes et al., 2010; Meuleman et al., 2013). Efforts have been made to identify DNA sequence elements required for association with the NL. Zullo et al. (2012) showed that specific DNA sequence motif, but not the transcriptional inactivity or chromosomal locations of the transgenes, was the primary determinant for positioning transgenes to the nuclear lamina. By fine scale mapping of LADs, they found that LADs are enriched for repeated GAGA motifs that bind to HDAC3 and Lap2 β and can direct lamina association (Zullo et al., 2012). Another study reported long stretches of A/T rich DNA in constitutive LADs (cLADs) by comparing mouse and human LADs (Meuleman et al., 2013).

Some studies also identified the elements that are required for positioning of specific genes to the nuclear periphery in *C. elegans* and mammalian cells. For example, promoter types and activities can influence gene positioning after transcriptional induction. During *C. elegans* embryo development, tissue-specific promoters *myo-3* (in muscle cells) and *pha-4* (in gut cells) could override the peripheral anchorage of large transgene arrays upon gene activation in the corresponding tissues (Meister et al., 2010). Interestingly, the subnuclear position of transgenes carrying both active and inactive promoters was determined solely by the active promoter (Meister et al., 2010), which implicated roles of transcription

machineries in gene positioning. Also in *C. elegans*, Heat Shock (HS) elements and HS-associated sites (HSAS) played a role in recruiting the *hsp-16.2* promoter to the nuclear envelope both before and after heat shock (Rohner et al., 2013). In mouse fibroblasts, the peripheral localization of BAC transgenes containing the β -globin gene remained unaltered upon deletion of the locus control region (*LCR*) (Bian et al., 2013). Instead, three peripheral targeting regions (PTRs) 80Kb upstream of the *LCR* were shown to be essential for the peripheral targeting of β -globin BAC transgenes (Bian et al., 2013). Interestingly, these PTRs resulted in preferential targeting of β -globin BAC transgenes toward the nuclear periphery rather than toward the PCH (Bian et al., 2013).

Several recent studies implicated epigenetic mechanisms such as repressive and active histone modifications in regulating gene positioning. In *C. elegans*, methylation of histone H3 Lysine9 (H3K9) was shown to be a molecular signal for anchoring genes to the nuclear lamina (Towbin et al., 2012). Similarly, H3K9me2 methyltransferase G9a was shown to promote nuclear lamina interactions with genomic regions in human cells (Kind et al., 2013). Simultaneous inhibition of G9a and Suv39H1/H2 (H3K9me3 methyltransferase) significantly reduced the peripheral localization of the endogenous human β -globin locus in human fibroblasts (Bian et al., 2013). Thus, differential compartmentalization of histone modifications may be one of the key determinants of gene positioning in metazoans. It should be noted that disrupting these epigenetic pathways did not necessarily lead to altered gene positioning for some other genes. For instance, H3K27 methylation is enriched at the *Mash1* promoter in ES cells. However, depletion of PRC2 complex (H3K27me3 methyltransferase), Suv39H1, G9a and Dnmt1, Dnmt3a and Dnmt3b (DNA methyltransferases) did not perturb the peripheral positioning of the *Mash1* gene (Williams et al., 2006). Therefore, alternative pathways may be responsible for locating the *Mash1* gene to the nuclear periphery in ES cells.

Mechanisms driving gene repositioning

There are several potential mechanisms underlying gene repositioning within the nucleus. It is conceivable that the dynamic motion of chromatin loci during interphase is a major factor driving gene repositioning (Ferrai et al., 2010). Indeed, ATP-dependent chromatin remodeling complexes (Neumann et al., 2012) and nuclear actin/myosin were both implicated in gene loci mobility and movement (Chuang et al., 2006; Hofmann et al., 2006). Transcription by the RNA polymerase holoenzyme, which is considered a powerful molecular motor, may also trigger chromatin movement (Marko and Poirier, 2003; Kouzine et al., 2004; Lanctôt et al., 2007).

INO80 nucleosome remodeling complex was shown to enhance the nuclear mobility of the *PHO5* gene in yeast

(Neumann et al., 2012). The increased mobility of the *PHO5* gene upon its activation by targeting the viral transcription activator VP16 was dependent on the ATPase activity of INO80 remodeler but independent of SWI/SNF or Gcn5 histone acetyltransferase (Neumann et al., 2012). Intriguingly, different transcriptional activation pathways of the same gene may result in distinct gene positions within the nucleus. For example, activation of the subtelomeric gene *HXX1* through glucose starvation was accompanied by increased associations with the NPC; however, activation of the same gene by targeting VP16 to its promoter led to its repositioning toward the nuclear interior (Taddei et al., 2006). One can imagine that if different transcription activation pathways recruit distinct regulatory complexes, the resulting interactions of these macromolecular complexes with the target gene loci could differentially modulate gene repositioning.

Nuclear actin and myosin I were shown to be essential for interphase chromosome mobility and gene positioning (Chuang et al., 2006; Hofmann et al., 2006; Dundr et al., 2007). Dundr et al. (2007) demonstrated that upon transcriptional activation, the U2 snRNA arrays repositioned toward the Cajal body by long-range (2–3 μ m) directed motion with apparent velocities of 0.1–0.2 μ m/min. Intriguingly, repositioning of the transgene to the Cajal body was disrupted when a dominant negative mutant of actin was expressed (Dundr et al., 2007). Expressing mutants of both nuclear myosin I NMI (E407V) and non-polymerizable actin (G13R) also blocked chromatin mobility (Chuang et al., 2006). In contrast, chromatin mobility was enhanced upon expression of the actin mutant (S14C) which stabilizes F-actin (Chuang et al., 2006). These data suggest that nuclear actin and myosin I may be involved in the directed motion of the gene loci and/or chromatin mobility in mammalian cells.

Spatial compartmentalization of regulatory factors within the cell nucleus may also play a role in gene repositioning. By high resolution imaging in mammalian cells, we have recently demonstrated that the nuclear periphery can be distinguished from the nuclear interior by the paucity of active histone modifications, in addition to the enrichment of repressive histone modifications (Fig. 3) (Yao et al., 2011; Wu and Yao, 2013). Interactions of the molecular machineries with their target genes may contribute to gene repositioning to the corresponding nuclear subcompartments (Ragoczy et al., 2006; Yao et al., 2011). For example, the *MyoD1* gene was repositioned toward the nuclear interior during muscle differentiation (Yao et al., 2011) and became accessible to TAF3, which was sequestered to the nuclear interior via interaction with H3K4me3 through its plant homeo domain (PHD) finger (Vermeulen et al., 2007; Yao et al., 2011). Similarly, β -globin locus moved from the nuclear periphery to the nuclear interior during erythrocyte differentiation when RNA Pol II foci became more restricted to the nuclear interior in differentiated cells (Ragoczy et al., 2006). These findings were reminiscent of previous observations where some co-regulated genes were colocalized in the nucleus and could

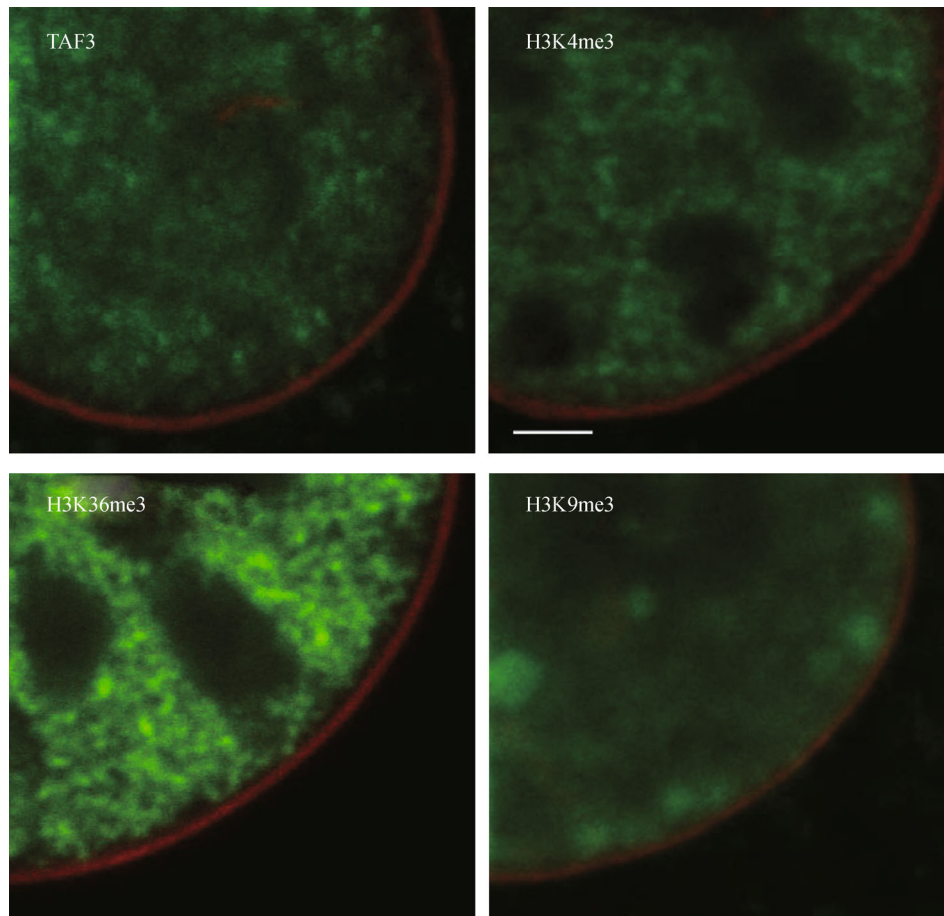


Figure 3 Spatial compartmentalization at the nuclear periphery. Immunofluorescence staining of TAF3 and histone modifications was performed in mouse C2C12 myoblasts. TAF3, H3K4me3, H3K36me3 (green) were all localized away from the nuclear periphery which was labeled by anti-Lamin B (red). The H3K9me3 mark was present at both the nuclear interior and the nuclear periphery. Scale bar: 2 μ m.

share the same transcription factories or nuclear speckles (Osborne et al., 2004; Osborne et al., 2007; Brown et al., 2008b; Lawrence and Clemson, 2008).

Genome wide mapping approaches to understand chromatin organization within the nucleus

Although cellular imaging provides substantial information on gene positioning and repositioning in the nucleus, optical microscopy cannot distinguish whether genomic loci are in molecular contact with the nuclear lamina or the NPCs (Takizawa et al., 2008b; de Wit and de Laat, 2012; Naumova et al., 2012). Genome-wide mapping techniques can provide high-resolution molecular maps of genomic interactions with nuclear landmarks and chromosome conformations (Simonis et al., 2007; van Steensel and Dekker, 2010; de Wit and de Laat, 2012).

A common technique used for mapping genome interactions with nuclear landmarks is the DNA adenine methyltransferase identification (DamID) assay. In this assay, a

fusion protein consisting of a protein of interest and the Dam enzyme was expressed in cells (van Steensel and Henikoff, 2000; Vogel et al., 2007). Adenines within the GATC motifs adjacent to the binding sites of the protein of interest were methylated by Dam in the fusion protein. The methylated DNA was subsequently amplified by methylation-specific PCR. PCR products were then hybridized to DNA microarrays (Vogel et al., 2007) or processed for next generation sequencing (NGS) (Wu and Yao, 2013).

Several genome-wide DamID maps of the nuclear envelope proteins including nuclear lamins and the NPC were successfully constructed in cells from several organisms including *S. pombe* (Steglich et al., 2012), *D. melanogaster* (Pickersgill et al., 2006; Kalverda and Fornerod, 2010; Kalverda et al., 2010), *Arabidopsis thaliana* (Germann et al., 2006), as well as mouse and human (Guelen et al., 2008; Peric-Hupkes et al., 2010). LADs covered about 40% of the genome and were 0.1 to 10 megabases (Mb) in size. Compared to non-LADs regions, the LAD regions were gene-poor, late-replicating, and transcriptionally less active (Pickersgill et al., 2006; Guelen et al., 2008; Wu and Yao, 2013). The boundaries of LADs were enriched with CTCF

binding sites, CpG islands, and promoters transcribing away from the LADs (Guelen et al., 2008). Some LADs were associated with the NL in a cell type-independent manner and may contribute to a basal chromosome architecture (Meuleman et al., 2013). These constitutive LADs were conserved between mouse and human and were characterized by long stretches of DNA of high A/T content (Meuleman et al., 2013). However, there are many local variations in LADs composition that are cell type-specific, suggesting that NL-chromatin interactions may regulate lineage commitment and differentiation (Peric-Hupkes et al., 2010; Luperchio et al., 2014). Recently, our laboratory constructed high resolution maps of lamina-associated domains called sequencing-based LADs (sLADs) that range from a few kilobases (Kb) to over 1 Mb in size. These high-resolution maps enabled the examination of NL associations within the structure of genes. For example, we have found that among active genes associated with the NL, NL-association levels were substantially lower around the transcription start sites (TSSs) than in the gene body (Wu and Yao, 2013).

Genomic regions interacting with the NPC were also mapped by chromatin immunoprecipitation (ChIP) in which the chromatin crosslinked to NPC components was mechanically sheared and immunoprecipitated using antibodies against Nucleoporins (Nups). The extracted DNA was then analyzed by PCR or microarray. In *S. cerevisiae* and *D. melanogaster*, Nups were preferentially associated with transcribed genes with Nucleoporin-Associated Regions (NARs) covering around 25% of the genome. The NARs were dominated by markers for active transcription like RNA polymerase II and histone H4K16 acetylation (Casolari et al., 2004; Capelson et al., 2010; Vaquerizas et al., 2010). However also in budding yeast, Nup93 ChIP revealed that it interacts with chromatin regions enriched with inactive genes and repressive histone modifications (Brown et al., 2008a). Additionally, loss of histone deacetylation led to large-scale changes in NPC-chromosome interactions (Brown et al., 2008a). Because Nups were also found to interact with genes inside the nucleoplasm, these genome-wide mapping data did not necessarily indicate the association of genes with the NPCs (Capelson et al., 2010; Kalverda et al., 2010; Vaquerizas et al., 2010). Consistent with microscopy analysis (Schermelleh et al., 2008), NARs did not overlap with LADs (Kalverda et al., 2010). Interestingly, enrichment of the insulator protein Su(Hw) near the NPC domain border may prevent the spreading of heterochromatin from LADs, thereby linking NPCs to chromatin insulator functions (Kalverda and Fornerod, 2010).

Another major nuclear landmark is the nucleolus, which is associated with genes encoding rRNA. Deep-sequencing of DNA isolated from purified nucleoli has provided genome-wide high-resolution maps and revealed nucleolar-associated domains (NADs) (van Koningsbruggen et al., 2010). NADs were enriched in AT-rich sequence elements, had a low gene density, and generally contained repressed genes. Interest-

ingly, a partial overlap between the NADs and LADs has been observed (Németh et al., 2010; van Koningsbruggen et al., 2010). Kind et al. using the ^{m6}A-Tracer technique detected some LADs became closely associated with nucleoli after mitosis of mother cells (Kind et al., 2013). This observation suggests that portions of the genome are stochastically shuffled between nucleoli and the NL during cell division.

Chromosome Conformation Capture (3C) and derivative methods have been employed to map long-range chromatin interactions both between pair-wise gene loci and at a genome-wide level (Dekker et al., 2002; Horike et al., 2005; Dostie et al., 2006; Simonis et al., 2006; Lieberman-Aiden et al., 2009; Splinter et al., 2011; Naumova et al., 2012). The initial step in 3C and 3C-derived methods involves fixation of chromatin with formaldehyde followed by digestion with a restriction enzyme that is 6 base pairs (bp) or a frequent cutter. The sticky ends of the cross-linked DNA fragments are then re-ligated under diluted conditions to promote ligations between cross-linked fragments. This allows the ligation of DNA fragments that are far away on the linear template but co-localized in space, thereby facilitating the identification of physical interactions of distal loci in the 3D nuclear space by PCR, microarray or high-throughput sequencing. The chromosome conformation capture-on-chip (4C) technology is best suited to examine the physical interactions between a specific locus and the rest of the genome. Using 4C it was shown that the active globin genes were associated with hundreds of other transcribed gene (Simonis et al., 2006; Schoenfelder et al., 2010). Similarly, inactive β -globin gene formed long-range interactions with other inactive chromosomal regions (Simonis et al., 2006). In *Drosophila*, 4C analysis revealed that Polycomb target genes were preferentially clustered (Tolhuis et al., 2011). Hi-C provides unbiased interaction maps of the whole genome and confirms the existence of chromosome territories and the spatial proximity among small, gene-rich chromosomes (Lieberman-Aiden et al., 2009).

Implications of gene positioning on transcriptional regulation

Previous cytological studies and gene tethering experiments demonstrated that for some genes, subnuclear positioning and transcription are functionally linked (Sexton et al., 2007; Takizawa et al., 2008b; Ferrai et al., 2010; Kind and van Steensel, 2010; Geyer et al., 2011; Deng and Blobel, 2013). Nonetheless, the mechanisms by which gene transcription is modulated by altering gene positioning, or by which gene positioning is modulated by altering transcriptional activities remain unclear. Some classical and recent studies attempted to address these questions.

Zink et al. (2004) showed that upon inhibiting transcription by 5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole (DRB) the transcriptionally active *CFTR* gene repositioned to the

nuclear periphery. On the other hand, targeting of the transcription activator VP16 induced the repositioning of tagged chromosomal loci to the nuclear interior (Tumbar and Belmont, 2001). These studies indicate that transcriptional activities were necessary and sufficient (in case of activation by VP16) for gene repositioning in mammalian cells. In *C. elegans*, heat shock factor (HSF-1), enzymatically active RNA Pol II and the THO/TREX component ENY-2 (*C. elegans* homolog of Sus1) were necessary to position *hsp-16.2* promoter transgenes to the nuclear periphery at both non-heat shock and heat shock conditions (Rohner et al., 2013). The authors proposed that promoter-bound Pol II or short Pol II-dependent transcripts—but not the abundant mRNA—is essential for NE localization, and that NPC association is enhanced by the transcription of nascent mRNA (Rohner et al., 2013). Identification of factors that can rescue the loss of gene localization to the NE upon RNAi knockdown of HSF-1 or ENY-2 will be helpful in future investigations.

Many previous observations support the notion that gene transcriptional status can be influenced by their subnuclear positions. A classic example is the trans-silencing of the *Brown* locus in *D. melanogaster* when it was repositioned to a heterochromatic region (Csink and Henikoff, 1996; Dernburg et al., 1996). Similarly, artificial tethering transgenes and endogenous genes to the nuclear periphery resulted in transcriptional repression accompanied by histone hypoacetylation (Andrulis et al., 1998; Finlan et al., 2008; Reddy et al., 2008). Nonetheless, the expression of many endogenous genes neighboring the tethered locus remained unchanged upon NL tethering (Finlan et al., 2008). Moreover, a transgene array tethered to the NL could be activated upon transcriptional induction (Kumaran and Spector, 2008). Therefore, genes may have different sensitivities to the dampening effect of the nuclear periphery on their expression, or there could be transcriptionally permissive microenvironments at the nuclear periphery. Further efforts should be made to describe how the nuclear periphery (such as association with the NL or NPC) regulates transcription of individual genes in metazoan cells, such as kinetics of activation and repression, Pol II density/distribution, elongation rates, etc.

In addition to modulating gene transcriptional activities, the peripheral localization of genes in yeast was also proposed to establish ‘transcriptional memory’ that promotes faster reactivation of genes. The endogenous *GAL* genes, *GAL* transgenes and the *INO1* gene maintained their peripheral nuclear localization even after transcription shutoff (Abruzzi et al., 2006; Brickner et al., 2007). Moreover, reactivation of recently repressed *GAL* genes was much faster than the initial gene activation and was dependent on the NPC component Nup2 (Brickner et al., 2007). These observations suggested the existence of ‘transcriptional memory’ at the nuclear periphery that confers a faster transcriptional activation of recently repressed genes. The histone variant H2A.Z (Brickner et al., 2007) and the chromatin remodeling complex

SWI/SNF (Kundu et al., 2007) were also found to be required for this apparent transcriptional memory. Whether such modes of transcription memory exist for NPC-associated genes in metazoans remain to be studied.

Future perspectives

Cell imaging and genome-wide analysis are excellent tools to characterize the 3D gene positioning and genome organization in the nucleus. Several important phenomena such as inter- and intra-chromosomal DNA looping and nuclear compartmentalization of histone modifications have been examined in convergence by cell imaging and genome analyses (Schoenfelder et al., 2010; Wu and Yao, 2013). The advent of studying single-cell genome organization will bridge the current gap between genomics and microscopy studies of chromosomes and cell nucleus (Nagano et al., 2013). Because chromatin mobility is the basis for the repositioning of a gene locus, live cell imaging and 3D tracking of gene loci to the sub-diffraction level will be important to understand the mechanisms of gene positioning and the effects of regulatory factors or a nuclear compartment (such as the nuclear periphery) on gene mobility in mammalian cells.

Artificially tethering genes to the nuclear periphery or inducing DNA looping has been a powerful approach to study their effects on gene expression (Finlan et al., 2008; Reddy et al., 2008; Deng et al., 2012). With the development of transcription imaging (Lionnet et al., 2011), more precise measurements can now be made to understand the effects of NL association and/or gene looping on transcription kinetics. Notably, chromatin mobility is higher in ES cells than in somatic cells (Mattout and Meshorer, 2010), which potentially allows rapidly inducing gene repositioning and may render ES cells a valuable model to study the effects of spatial gene positioning.

Genetic studies in model organisms have identified factors that can influence the subnuclear positioning of specific genes. Gain-of-function studies such as introducing tether proteins may be helpful in identifying the direct regulators of gene positioning. Unlike gene expression levels that are often measured by amplitudes, subnuclear gene positioning appears to be a cellular property largely modulated in frequency. Changes in gene positioning are generally identified as changes in the proportion of cells where genes are positioned next to a nuclear landmark. Simultaneous live cell imaging in a large number of cells during rapid gene repositioning will be important in understanding the regulation of this process.

Acknowledgements

We thank Brennan Olson and Duc Nguyen (Yale University School of Medicine) for critical reading and editing of the manuscript. This work was supported by the startup funding from Yale School of

Medicine, a Scientist development grant from American Heart Association (12SDG11630031), and a seed grant from State of Connecticut Stem Cell Research Program (13-SCA-Yale-15).

Compliance with ethics guidelines

Nidhi Vishnoi and Jie Yao declare that they have no conflict of interest. This manuscript is a review and does not involve a research protocol requiring approval by the relevant institutional review boards or ethics committees.

Abbreviations

3D: three dimensional
 NL: nuclear lamina
 NPC: nuclear pore complexes
 PML: promyelocytic leukemia
 PCH: pericentromeric heterochromatin
 CSN: casein gene cluste
 ES: embryonic stem
 MEF: mouse embryonic fibroblast
 iPS: induced pluripotent stem
 FISH: fluorescence *in situ* hybridization
lacO/lacI: *lac* operator/*lac* repressor
 DIG: digoxigenin
 ZFN: zinc-finger nucleases
 TALEN: transcription activator-like effector nuclease
 CRISPR: clustered regulatory interspaced short palindromic repeats
 LAD: lamin associated chromatin domain
^{m6}A: adenine-6-methylation
 cLAD: constitutive LAD
 LCR: locus control region
 PTR: peripheral targeting region
 HS: heat shock
 HSAS: HS-associated sites
 H3K9: H3 Lysine9
 UTR: untranslated region
 PHD: plant homeo domain
 DamID: DNA adenine methyltransferase identification
 Dam: DNA adenine methyltransferase
 NGS: next generation sequencing
 Mb: megabases
 sLAD: sequencing-based LAD
 TSS: transcription start site
 Kb: kilobases
 ChIP: chromatin immunoprecipitation
 Nups: nucleoporins
 NAR: nucleoporin-associated region
 NAD: nucleolar-associated domains
 3C: chromosome conformation capture
 4C: chromosome conformation capture-on-chip
 DRB: 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole
 TSA: trichostatin A
 ES cells: embryonic stem cells

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