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Locking the genome: nuclear organization and cell fate

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The differentiation of pluripotent or totipotent cells into various differentiated cell types is accompanied by a restriction of gene expression patterns, alteration in histone and DNA methylation, and changes in the gross nuclear organization of eu- and heterochromatic domains. Several recent studies have coupled genome-wide mapping of histone modifications with changes in gene expression. Other studies have examined changes in the subnuclear positioning of tissue-specific genes upon transcriptional induction or repression. Here we summarize intriguing correlations of the three phenomena, which suggest that in some cases causal relationships may exist.

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In the last decade, interest in embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) has increased sharply due to their potential for medical application [1•]. A number of studies link specific chromatin modifications and the spatial organization of the genome with cellular pluripotency, defined as the capacity of ESCs and iPSCs to generate differentiated tissues. Here we review recent data on chromatin and DNA modifications and their relationship to nuclear organization during cell fate acquisition and differentiation. We explore evidence that argues for a causal link between the 3D organization of the genome and cell type commitment in model organisms.

Genomic marks: pluripotent versus differentiated epigenomic landscapes

During the course of development and differentiation, cells acquire specific fates by altering their transcriptional profiles. Not surprisingly, differentiation also correlates with changes in the distribution of epigenetic marks. These changes alter the repartition and abundance of both repressive and active histone tail modifications, DNA methylation and the accessibility of transcription factor binding sites to their ligands [2–6].

Genome-wide methods such as ChIP-chip or ChIP-seq have begun to map systematically the human and mouse epigenomes of both undifferentiated (e.g. ESCs) and differentiated cells, and have followed changes in these marks during induced differentiation. A frequently used model system exploits the differentiation of mouse ESCs first into neural progenitors (neural precursor cells, NPCs) and then into various neural cell types [7]. Alternatively, ESCs can be compared with independently obtained differentiated cell lines. Although a complete epigenomic analysis through a differentiation pathway is still lacking, some general conclusions can be extracted from these studies.

One uncontested change is that DNA methylation generally increases on tissue-specific promoters that become silenced during tissue differentiation; intriguingly, in the committed precursor stage these *de novo* methylation targets often are bivalent for active and repressive marks (i.e. carry both active H3K4 and silent H3K27 methylation) [8]. Similar bivalent promoters have been found in zebrafish embryos at the onset of genome activation [9•]. A second conclusion from these studies is that the global amount of the repressive Polycomb-associated mark H3K27 trimethylation does not change significantly during the differentiation of ESCs to neuronal cells, even though H3K27^{me3} levels fluctuate a great deal at individual promoters. Hundreds of promoters gain this Polycomb-deposited mark and hundreds also lose it during the transitions from ESC to committed precursor (NPCs) and from NPC to differentiated neuron [10]. In many cases a loss of H3K27^{me3} coincides with loss of bivalency. Third, in zebrafish, mouse and human pluripotent cells many promoters appear to be marked with H3K4 trimethylation, even in the absence of detectable polymerase [9•,11–14], Upon differentiation, on the other hand, most promoters with H3K4 trimethylation become transcriptionally active. Importantly, the methylation of both H3K27 and H3K4 is enriched within CpG islands, providing one explanation for the overlap. It has been shown that the CpG-binding protein Cfp1 induces H3K4 trimethylation at CpG islands that lack DNA methylation, even in the absence of a promoter [15]. On the repressive side, many Polycomb-binding sites reside within hyperconserved CpG islands, and introduction of exogenous CpG sequences is sufficient to recruit PRC2 [8,14,16,17].

There is less consensus on the behavior of the histone modification associated with constitutive heterochromatin;

that is, it is unclear whether the amount of histone H3K9 dimethylation and trimethylation increases during differentiation of ESCs [18,19]. In many organisms this mark is associated with the repetitive DNA at centromeres, and would not be expected to change with differentiation. However, H3K9^{me} is also associated with the binding of heterochromatin protein 1 variants (HP-1 α, β, or γ), which are linked to both gene repression and transcriptional elongation during tissue development [20–22]. Immunofluorescence studies showed increase in the number of HP1-α containing foci and the intensity of H3K9^{me} staining during conversion of mouse ESCs to NPCs [23]. However, genome-wide changes in histone marks were not quantitatively as large one might have expected given the major reorganization of the genome that occurs during differentiation (see below).

Multiple classes of chromatin during differentiation

Many of the chromatin marks analyzed in the mapping studies are spatially segregated in domains of similarly marked chromatin within the nucleus. Although there is no comprehensive study of the nuclear morphology of histone marks as yet, anecdotal evidence shows that the shape, number and size of such chromatin domains often change with differentiation [24–27]. Indeed, electron-dense and DAPI-staining heterochromatin foci appear to increase during the course of differentiation [24,28,29•].

For decades, electron microscopic images of osmium-stained nuclei allowed one to distinguish euchromatin (lightly stained) from heterochromatin (darkly stained) with the latter often tightly sequestered by the inner nuclear envelope [30]. Two recent studies, using principal component analysis and/or Hidden Markov Models (HMMs) to define chromatin classes on the basis of nonhistone protein enrichment [31••] or enrichment of specific histone marks [32••], now suggest that this binary classification scheme is too simple to describe the relevant classes of chromatin. At least five distinct types of chromatin, classified by the abundance of specific nonhistone binding factors, were identified in *Drosophila* Kc167 cells [31••]. This remarkable study by the van Steensel laboratory used fusions of the bacterial DNA methylase (Dam) to 53 different chromatin associated factors, to score their distribution over the genome through the quantitative mapping of adenine methylation (DamID [33]). They identified three distinct classes of silent chromatin, namely, the simple-repeat associated HP1-binding chromatin found at centromeres, the H1-associated and lamin-associated chromatin that is enriched for silent tissue-specific genes, and finally silent domains enriched for Polycomb [31••]. They further identified two classes of transcriptionally active chromatin: one distinguished by an enrichment for H3K36 methylation and its ligand, Mrg15, and a second that is replicated very early and is enriched for large regulatory protein complexes.

A distinct HMM approach was applied to histone modifications mapped in human CD4+ T cells [32••,34]. This study also identified five classes of euchromatin and heterochromatin, and in contrast to the van Steensel study, could distinguish upstream regulatory sequences from coding regions based on their histone mark profiles [32••]. These classifications will surely be exploited in future studies that characterize chromatin changes during cellular differentiation. Even if a few more chromatin types are found, the fact that a limited number of categories of chromatin can be robustly identified by HMM analysis, argues that large domains of the genome share structural characteristics.

Consistent with the notion that at least one class of heterochromatin accumulates at the nuclear rim during differentiation, the progressive association of repressed pluripotency genes and silent tissue-specific genes with the nuclear lamin has been scored during fly differentiation or as neurons are generated from ESCs [35••,36]. Analysis of the genomic sequences lying close to the nuclear envelope has shown that lamin-interacting domains tend to be transcriptionally silent [36,37]. In both flies and mouse cells the overall percentage of the genome attached to lamin is large (40–48% of the probed genome) [36,37], yet only about 1000 of over 17 000 genes scored (12%) show a significant increase in lamin association during the mouse ESC to neuronal differentiation [35••]. Importantly, these 1000 are enriched for pluripotency genes, which become repressed as cells differentiate, and silent non-neuronal tissue-specific genes. Nonetheless, 30% of the genes that became lamin-bound did not change in expression, indicating that the nuclear periphery does not necessarily impose transcriptional repression [38]. In the other direction the correlation was somewhat more robust: many of the genes that were released from the lamina upon differentiation were shown to be 'unlocked' or 'open' for lineage-specific transcription, even though active transcription might only occur much later. In conclusion, DamID profiles on *in vitro* neuronal differentiation showed surprisingly few changes in lamin-associated domains (LADs), despite the seemingly major increase in chromatin at the nuclear rim in differentiated cells [24,28]. Moreover, although genes shifted to the nuclear envelope are enriched for repressed loci, it is clear that gene repression and peripheral sequestration are not fully congruent.

Increase of genome compaction correlates with decrease of genome plasticity and stabilization of cell fate

While tissue culture cells and *in vitro* differentiation systems have opened the door to genome-wide chromatin studies, recent work in the nematode *Caenorhabditis*

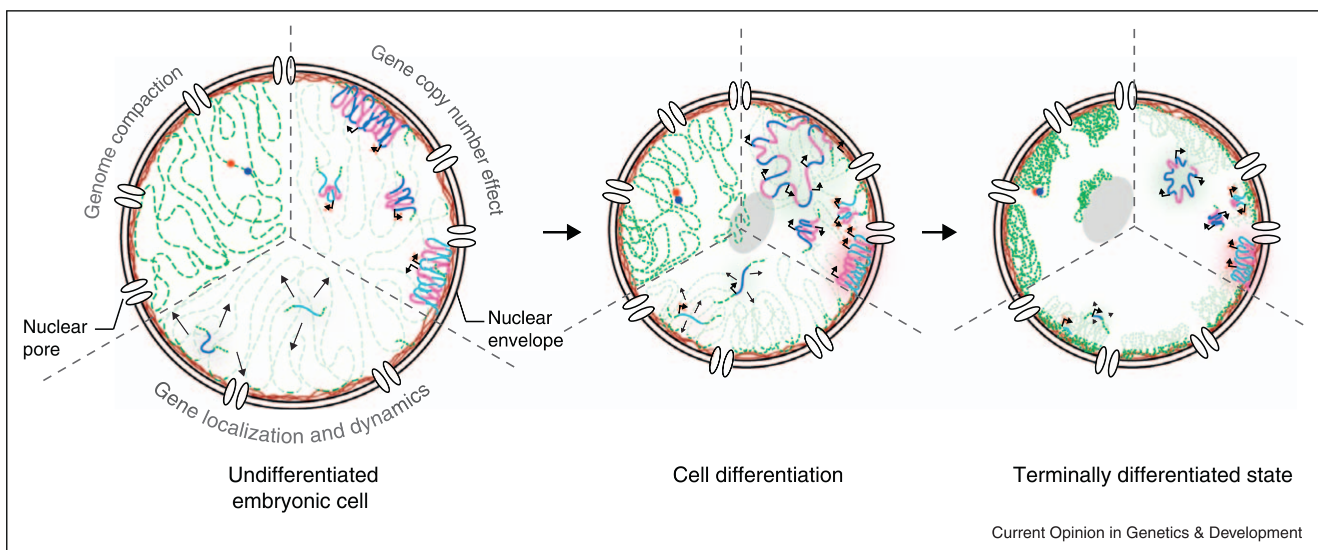
elegans has allowed one to explore how changes in nuclear organization influence differentiation events on an organismal level. Consistent with the lack of centromeric repeats and pericentric heterochromatin, *C. elegans* embryos show little electron-dense chromatin at the earliest stages of development [39]. Yet, compact, transcriptionally silent heterochromatin appears progressively as cells differentiate in late embryonic stages and is found enriched at the nuclear periphery or close to the nucleolus (Figure 1 and [39]). Similarly, mouse ESCs were also reported to have low levels of heterochromatin, which increase during differentiation [24,28,29•]. In worms and mice, this accumulation of heterochromatin correlates with a loss of both pluripotency and the capacity to be reprogrammed to another cell fate. Inactivation of Polycomb in worms both prolongs both developmental plasticity and an open chromatin configuration [40••]. While the role of Polycomb for chromatin compaction in ES cells has not been investigated, inactivation of Polycomb interferes with differentiation, similar to worms [41].

Nuclear reprogramming to an alternative cell fate can be readily induced during worm development by the ectopic expression of 'selector genes' or 'master regulatory' transcription factors. If artificially induced at early stages of worm development, these master regulators are able to transform an entire embryo into a single tissue [42–44]. The efficiency of this transformation, or the degree of 'cell plasticity', decreases as worms develop and its cells acquire specific cell fates [40••]. A similar drop in the efficiency of reprogramming has also been characterized in mouse by the use of nuclear transplantation. As early as the blastocyst stage, mouse nuclei injected into enucleated oocytes or into early embryos showed a reduced ability to support organismal growth [45]. This same inefficiency for dedifferentiation or reprogramming is manifest when nuclei from differentiated tissues are fused with ESCs [46].

This loss of developmental plasticity correlates with a compaction of genomic chromatin — not only into domains associated with H3K27 methylation [40••], but also with the nuclear lamina [35••]. Consistent with these observations, chromosomes 6 and 8 appeared to undergo compaction during human ESC differentiation [25]. Electron microscopic studies suggest that the mouse embryonic genome similarly undergoes progressive compaction between the one cell stage and E5.5 [29•].

Given this apparent reduction of genome volume, it is relevant to ask what fraction of nuclear space is occupied by DNA, and how this changes through development. Using serial block face electron microscopy, a technique that allows reconstruction of entire nuclei at electron microscopy resolution, differentiated hepatocytes and

Figure 1



Major nuclear reorganization events over the course of cell fate acquisition. During differentiation, the genome gets globally compacted (upper left third), with clusters of compact chromatin being located close to the lamina (red meshwork) at the nuclear periphery or the nucleolus (light grey), leaving gaps or channels between large chromatin masses. Based on *C. elegans* studies, gene copy number is seen to influence nuclear localization (upper right third). In embryonic cells, heterochromatic high copy number repeats are located at the nuclear rim while low-copy repeats do not show preferential localization. During differentiation, activation of promoters is able to overcome the anchoring at the nuclear periphery. Preferential localization at the nuclear lamina is observed for silent tissue-specific promoters, while active tissue-specific promoters are more likely to be internal. Gene and chromatin associated dynamics decrease progressively as cells differentiate (lower third). This is correlated to the disappearance of some remodelers and the increase of anchored compact chromatin domains.

endothelial nuclei were estimated to have between 40 and 60% of their volume free of DNA [47]. The compaction of the genome through differentiation may leave space for transcription, splicing, replication and repair events in 'empty zones' or 'perichromatin' channels. Indeed, transcription occurs primarily external to dense chromatin territories and can be visualized by the looping out of activated genes from the bulk of a chromosomal domain into less dense interchromosomal space [48,49].

What could be the function of this genomic subcompartmentalization into domains of compacted heterochromatic and more open euchromatin? One hypothesis is that compartmentalization would reduce the complexity of the genome by hiding a number of possible binding sites for transcription factors in silenced heterochromatin. This is particularly important for higher eukaryotes in which most of the genome is non-coding. Alternatively, the sequestration of inactive chromatin may by default help target the transcription machinery to sites of transcription [5]. Finally, the formation of heterochromatin clusters could sequester general repressors from promiscuous binding and repression as shown in yeast [50]. All three hypotheses share the virtue of reinforcing cell fate restrictions. In contrast, gene induction events leading to cell fate changes appear to be more stochastic.

In support of the model that transcription maintains an open chromatin state in undifferentiated cells, a microarray analysis suggested that ESCs have widespread transcription throughout their genomes, including intergenic sequences, whereas differentiated cells did not [28]. This finding, however, has been challenged by recent RNA-seq experiments which suggest that transcription is largely confined to annotated genes and is not widely different between ESCs and differentiated cells ([51] and D Schuebeler, personal communication). Clearly this topic merits further analysis. Intriguingly, chemical inhibition of heterochromatin formation by the histone deacetylase inhibitor TSA was found to impair ES cell differentiation [52], and both TSA and other chemicals that interfere with heterochromatin structure were shown to improve the efficiency of iPSCs generation (from 0.001% to 0.15%) when dedifferentiation is induced by a subset of the Yamanaka factors in differentiated cells [53,54].

Reorganizing chromosomes and genes in three dimensions

A small number of studies have directly examined the subnuclear organization of genes and chromosomes during mouse ESC differentiation. From the evidence available it seems that most chromosomes do not change their radial positioning during differentiation [27,55]. This suggests either that radial positioning is established as early as the stage represented by ESCs, or that position depends primarily on gene density and not on activity [27,56]. Nonetheless, some mouse chromosomes do show some degree of reorganization during differentiation. For example, centromeres are found more frequently associated with the nuclear rim or the nucleolus after differentiation [27,55]. Similarly, the silent X chromosome in female cells relocates toward the nuclear periphery when female hESCs are induced to differentiate [27]. Still, global chromosome positioning is unlikely to be a major regulatory feature in development, since developmentally regulated genes can be translocated or expressed ectopically with only minor effects on development.

In attempts to correlate chromosome position with the regulation of single genes during differentiation, genes encoding the pluripotency factors *NANOG* and *OCT3/4* have been a major focus. These genes, together with *SOX2*, *c-MYC* and *KLF4*, are highly expressed in ESCs, and their overexpression in differentiated cells can induce dedifferentiation and a pluripotent state (iPS cells) [57,58]. Consistent with results from other genes, the *NANOG* gene on the short arm of chromosome 12, gets repositioned upon repression, moving from the nuclear center in hESCs to the nuclear periphery in lymphoblastoid cells (hLCLs). Other studies documented the inverse switch for nonpluripotency genes: the β-globin gene gets relocated internally upon activation during erythroid differentiation, as does the proneural gene *Mash1* upon activation [59,60]. The monoallelically expressed astrocyte-specific *GFAP* gene also shows a slight inward shift, which again correlates with activity (internally active and peripherally inactive) [61]. Finally, the complete *Hox* locus was seen to reposition upon activation, by looping away from its chromosome territory [48,49]. These observations are anecdotal, however, and exceptions also exist: for example, the *OCT4* pluripotency gene, in contrast to *NANOG*, does not show changes in radial positioning upon differentiation [25,55]. This may reflect the fact that *OCT4* is situated much further from the edge of its chromosomal territory in LCLs than *NANOG* [55].

Unfortunately, these correlative data do not address what drives relocation nor do they prove that a shift in position is necessary to ensure gene activity. For this the *C. elegans* system proved more adept: by monitoring transgene insertions of different size, it could be shown that two major parameters dictate gene positioning *in vivo*: the size of a repetitive array and the transcriptional status of developmentally regulated promoters (Figure 1 and [62••]). Silent promoters in low-copy-number are randomly localized in undifferentiated embryonic cell nuclei, while the same sequence integrated as a high copy number array is recruited to the nuclear periphery [62••,63]. This copy-number dependence is correlated with the presence of inactive chromatin marks, H3K9^{me3} and H3K27^{me3}, although neither modification is sufficient for perinuclear anchoring, as they are found in foci

throughout the nucleus. On a genome-wide level, recent modENCODE data have shown a striking correlation in worms between peripheral localization, high repeat density and H3K9 methylation [64,65•,66•]. Similarly, LADs are associated with H3K9^{me2} and H3K27^{me3} histone marks in mammalian cells [37].

As differentiation progresses in *C. elegans*, tissue-specific genes become positioned, driven by the activity of their promoters, independently of copy number (Figure 1 and [62••]). This developmental control of promoter position is strongly reminiscent of the hypothesis drawn from differences in nuclear organization between ESCs and differentiated cells; that is, heterochromatin was seen to accumulate at the nuclear periphery [28] and chromosomes became increasingly compacted as cells acquired a differentiated cell fate [25]. Similar to mammalian cells, relocalization and decompaction could be correlated with the binding of master regulatory transcription factors [62••,67]. As proposed for mammalian cells [60,61,68,69], the acquisition of an altered nuclear organization, following from an altered chromatin structure, could be triggered by the opening of promoter domains by a master regulator [62••,67]. However, the nuclear lamina-associated protein emerin could impede the DNA binding and decompaction activities of the endoderm-determining factor PHA-4 in a subpopulation of embryonic cells [67], suggesting a tug-of-war between repressive activities associated with the lamina and activation by tissue-determining transcription factors.

Pluripotent chromatin: moving genes and proteins to keep all options open

Further evidence that the chromatin structure in ESCs is different from that in differentiated cells arose from fluorescence recovery after photobleaching (FRAP) experiments carried out with GFP-tagged nuclear proteins. GFP fusions to core histones H2A and H3, linker histone H1 and HP1 all showed higher turnover rates in ESCs than in committed neuronal precursor cells [70]. This suggests that nucleosome stability changes during differentiation, yet the removal of a histone chaperone, HirA had the surprising effect that ESCs differentiated faster, and not more slowly. Thus a simple correlation of higher turnover rates and increased pluripotency cannot be drawn, although decreased exchange of the linker histone H1 appeared to inhibit NPC cell fate acquisition, consistent with a reduction in cell plasticity [70].

The enhanced turnover of chromatin-bound proteins may also in part reflect the action of ATP-dependent chromatin remodeling. Chromatin remodelers are a family of multiprotein complexes containing an AAA+ ATPases domain, which is able to reposition or remove nucleosomes or subcomplexes of histones. Their activities facilitate both the induction and the repression of genes [71]. A number of remodelers have been shown to be essential for the maintenance of pluripotency, while others have been implicated in cellular transformation, with genes for specific subunits being identified as tumor suppressor genes [28,72–75]. For example, the chromatin remodeling factor Chd1 promotes open chromatin and a pluripotent state in mouse ES and iPS cells [75]. Moreover, it has been shown that some remodeling complexes are expressed uniquely in undifferentiated cells and are essential for pluripotent state maintenance, although their mechanism of action and target genes is still unclear [72,73]. Thus, much remains to be resolved as to how chromatin remodelers contribute to pluripotent states [28,72–75].

The effect of removing chromatin remodelers has been studied in yeast, since none is absolutely essential for vegetative growth on rich media. This redundancy suggests that no single nucleosome remodeler will be sufficient to induce pluripotency, nor is one likely to be essential for a specific differentiation pathway. Rather they can be considered to be instruments that ‘grease the wheels’ of change from one state to another. This may be achieved by facilitating the binding of transcription factors to their cognate sites, or by promoting the removal of certain histone variants or modified forms. With this perspective it is likely that the loss of a remodeler might limit access of tissue-specific transcription factors to their cognate sites, thus indirectly promoting the heterochromatinization of adjacent genes, to restrict genome-wide transcription of uncommitted cells [28].

In yeast, an unexpected effect of remodelers was monitored when they were targeted to a fluorescently tagged genomic locus: they were seen to increase gross chromatin mobility as monitored with time lapse microscopy, without changing its transcription level (F Neumann, SM Gasser, personal communication). In this context it is plausible that chromatin remodelers play a key role in the repositioning of genes and chromosomes during the transition from a pluripotent to a restricted potency state coincident with cell type differentiation [76]. This gene mobility, like histone eviction or repositioning, is an active process that requires ATP and responds to nutrient levels [77]. The space that a given locus can explore inside the nucleus is restrained by its physical link to a chromosome [78]. This constraint is further reinforced if the locus is located close to sequences which are anchored to nuclear structures, like the nuclear envelope or the nucleolus [78,79]. Thus it is probable, although not yet proven, that the relocalization of activated promoters is driven by chromatin remodeling.

In conclusion, systematic mapping techniques over the course of development have revealed relatively few large-scale changes in comparison with the extensive reorganization of specific chromatin loci inside the nucleus,

which can be observed microscopically. A careful comparative study using both approaches will be important to understand both sets of data. Only by characterizing the molecular players involved in genome three-dimensional reorganization, will we be able to understand how the spatial arrangement of genes impacts differentiation and the maintenance of cell fate.

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