L1.2

Nuclear organization

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Nuclear organization: taking a position on gene expression

Pamela K Geyer, Michael W Vitalini and Lori L Wallrath

Textbook

Eukaryotic genomes are divided into chromosomes that occupy defined regions or territories within the nucleus. These chromosome territories (CTs) are arranged based on the transcriptional activity and chromatin landscape of domains. In general, transcriptionally silent domains reside at the nuclear periphery, whereas active domains locate within the interior. Changes in nuclear position are observed for stress-induced and developmentally regulated tissue-specific genes. Upon activation, these genes move away from a CT to inter-chromosomal space containing nuclear bodies enriched in gene expression machinery. Gene activation is not always accompanied by movement, as positioning is dictated by many determinants, including gene structure and the local genomic environment. Collectively, tissue-specific nuclear organization results from a culmination of inputs that result in proper transcriptional regulation.

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chemical cross-linking and massive parallel sequencing to define genome-wide relationships [3–5]. Results from these studies suggest that the genome is arranged as inter-digitated CTs rather than randomly inter-twined chromosomes [6]. Emerging from these investigations is a picture of the nucleus as an ordered organelle; the consequences of this organization are just being realized.

Nuclear organization during differentiation

Studies have linked nuclear organization to cellular differentiation. Cultured pluripotent mouse embryonic stem (ES) cells possess dispersed chromatin with limited compaction. Upon differentiation, they show changes in chromatin structure that include large-scale compaction of genomic domains [7]. Consistent with these findings, embryonic development proceeds from a single cell embryo possessing a 'featureless' nucleus with dispersed chromatin, to differentiated cells possessing nuclei with peripherally located compact chromatin domains [8]. Interestingly, an extended and dispersed chromatin meshwork was identified in the eight-cell epiblast, reminiscent of nuclear structures defined in cultured ES cells. In contrast to the 'open' chromatin structure in the epiblast nuclei,

Geyer 2011 – 1st paragraph (yesterday, L1.1)

- chromosomes are confined to specific three-dimensional regions of the nucleus called chromosome territories (CTs)
- Gene-poor regions and silenced genes are frequently found in the nuclear periphery, a similar location to that of heterochromatin
- Gene-rich regions and active genes map to the nuclear interior.
- The genome is arranged as interdigitated CTs rather than randomly intertwined chromosomes

Geyer 2011 – 2nd paragraph

Nuclear organization during differentiation

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Transcriptionally active genes contribute to cell-type

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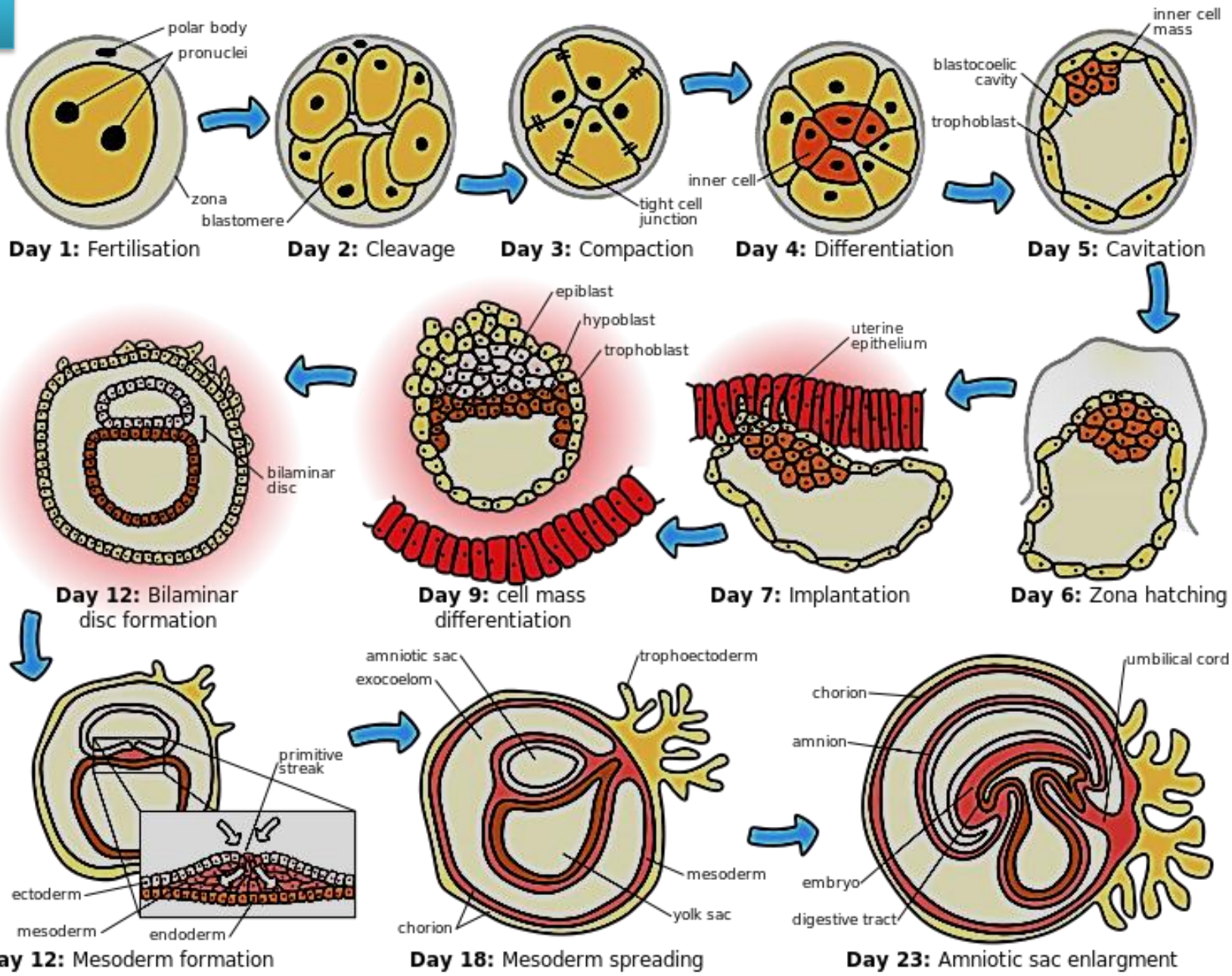
Geyer et al., 2011

wikipedia


polar body
pronuclei
zona
blastomere
tight cell junction
inner cell
blastocoelic cavity
trophoblast
inner cell mass
Day 1: Fertilisation
Day 2: Cleavage
Day 3: Compaction
Day 4: Differentiation
Day 5: Cavitation
Day 6: Zona hatching
uterine epithelium
Day 7: Implantation
epiblast
hypoblast
trophoblast
Day 9: cell mass differentiation
bilaminar disc
Day 12: Bilaminar disc formation
primitive streak
ectoderm
mesoderm
endoderm
Day 12: Mesoderm formation
amniotic sac
exocoelom
trophoectoderm
mesoderm
yolk sac
chorion
Day 18: Mesoderm spreading
chorion
amnion
umbilical cord
embryo
digestive tract
Day 23: Amniotic sac enlargment

These investigations indicate that global changes in gene expression correlate with chromatin reorganization, which plays a role in lineage restriction during development.

Geyer et al., 2011

Using transgenes that produced integrated arrays of different size, a hierarchy of subnuclear localization signals was uncovered [9••]. Large arrays produced 'heterochromatic' domains that were anchored to the nuclear periphery, whereas, smaller arrays localized within the nuclear interior. The spatial dynamics of genes within the arrays depended upon properties of the transgene promoter. Arrays with developmentally regulated genes relocated from the periphery to the interior in appropriate cell types. Before relocalization, markers of active transcrip-

Geyer et al., 2011

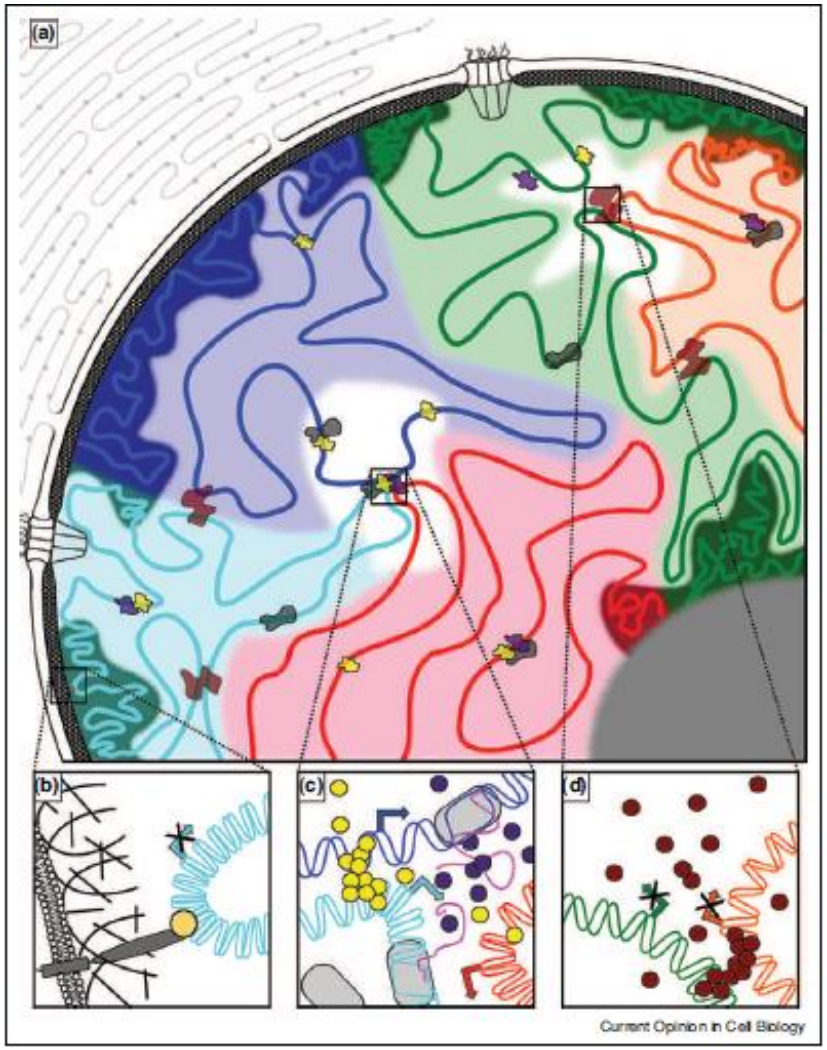
from the periphery. Collectively, these studies demonstrated that tissue-specific promoters are major determinants of nuclear organization during development.

Geyer et al., 2011

Peripheral localization of heterochromatin depends on proteins in the nuclear lamina, a network of proteins that line the inner side of the NE (Figure 1a and b) [10•]. Genomic sites that contact the nuclear lamina have been mapped by DamID [11] and are called lamina-associated domains (LADs) [12]. LADs range in size from tens of kilobases to several megabases, are relatively gene poor, have low transcriptional activity and are enriched in repressive chromatin marks. LADs make up nearly 40% of

Dam ID

Geyer et al., 2011



Geyer et al., 2011

The role of nuclear lamina in gene regulation

Peripheral localization of heterochromatin depends on proteins in the nuclear lamina, a network of proteins that line the inner side of the NE (Figure 1a and b)

LADs = Lamina-Associated Domains

Parts of the chromosomes that are attached to the nuclear lamina by

1) direct interaction ?

or

2) Nucleosome –mediated interactions
mediated by Lamina components or other associated proteins

LADs make up nearly 40% of human and mouse genomes

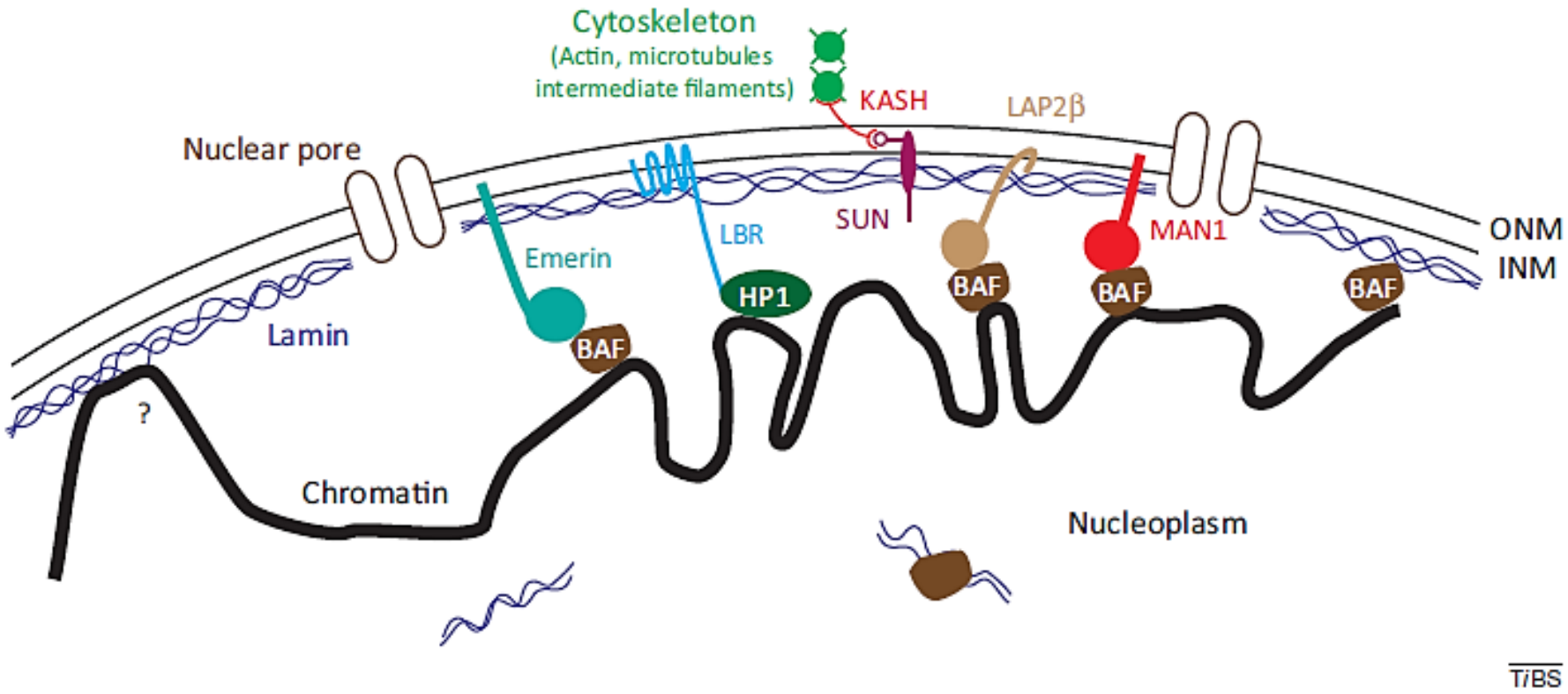
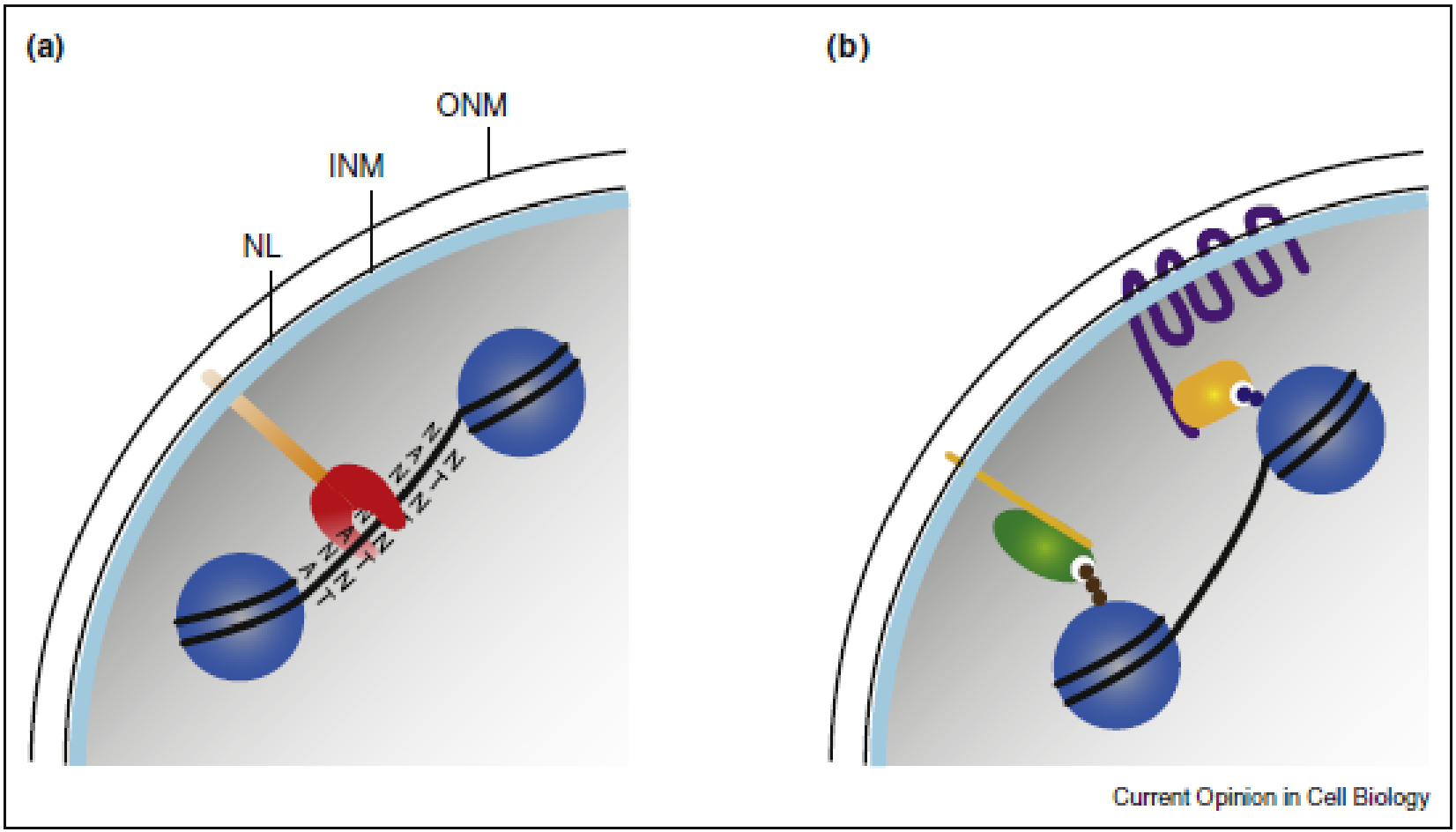


Figure 1. Schematic view of the nuclear envelope (NE). Lamins form a meshwork of intermediate filament proteins underlying the inner nuclear membrane (INM), where they interact with several distinct classes of transmembrane proteins. One class contains the NE-associated proteins Emerin, LAP2b (lamina-associated polypeptide 2b) and MAN1 (target of MAN autoimmune antibody 1), which interact via a conserved LEM (Lamina-associated-polypeptide, Emerin and MAN1)-related domain with the small protein barrier-to-autointegration factor (BAF), which has affinity for chromatin. A second example is the lamin B receptor (LBR), which contains seven transmembrane domains that span the INM. It interacts directly with the chromatin binding protein HP1 or in mammals with H4K20me through a Tudor domain. Finally, SUN (Sad1–UNC84 homology) domain proteins span the INM, and interact with lamins on the nucleoplasmic side and with KASH (Klarsicht/ANC-1/Syne Homology) domain proteins in the perinuclear space. KASH proteins (called Nesprins in human) span the outer nuclear membrane (ONM) and contact the cytoskeleton. Up to 10% of lamins and BAF are also found in the nucleoplasm in some cells.

Towbin et al., Trends in Biochem Sci., 38:356-363, 2013



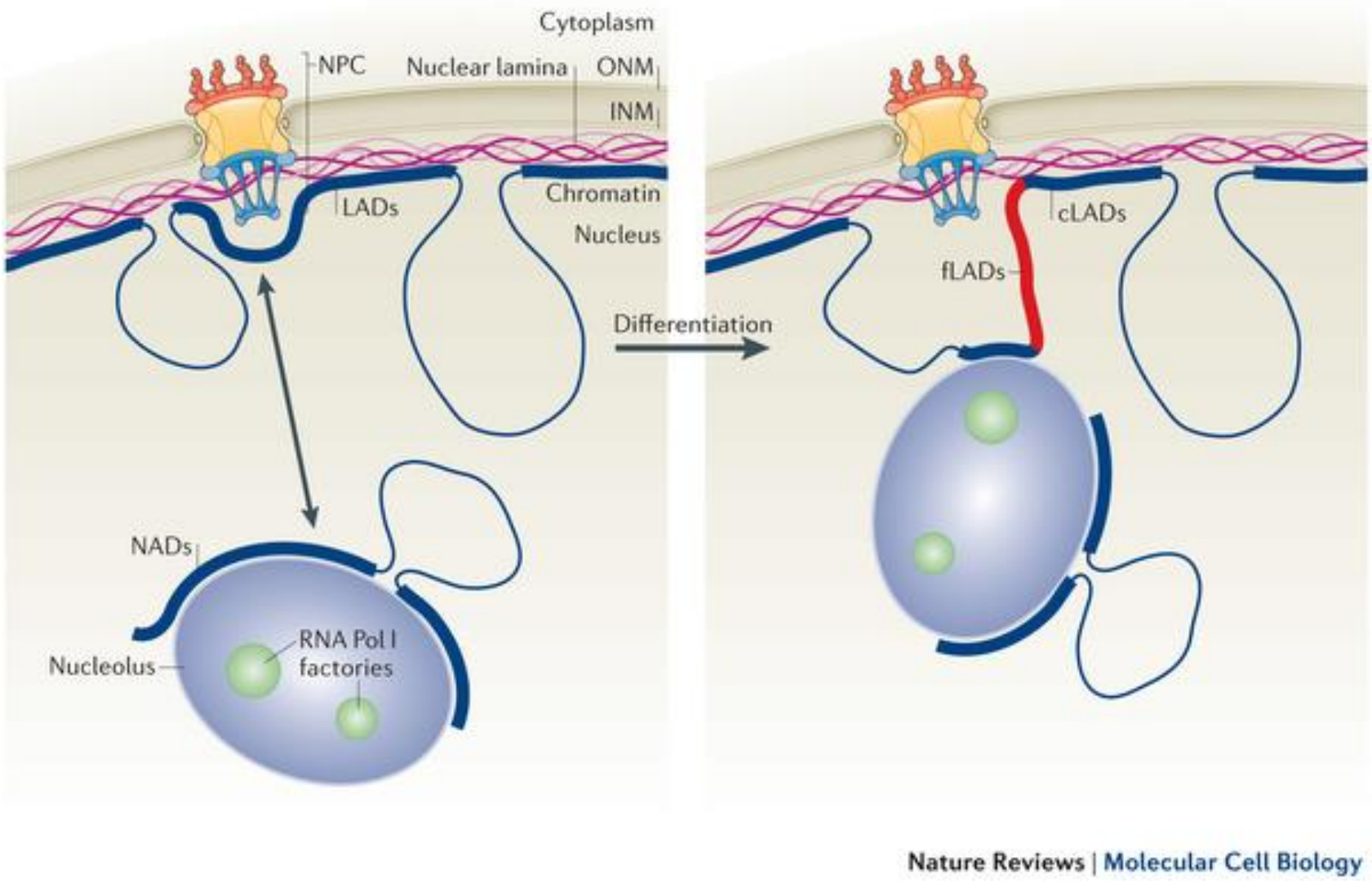
Cartoon model depicting two possible non-exclusive mechanisms responsible for the tethering of LADs to the NL. (a) DNA-binding factors anchored to the NL may recognize specific sequence motifs enriched in LADs; (b) Certain proteins interacting with the NL may bind specific histone modifications, such as H3K9 methylation. INM = inner nuclear membrane; ONM = outer nuclear membrane.

Amendola & van Steensel, Curr. Op. Cell Biol., 28:61–68, 2014

Another location of inactive (heterochromatic) loci is at the nucleolar perphery

These domains are called **NADs** (Nucleolus-associated domains)

Dynamic studies have shown that after mitosis LADs re-distribute in part. Some LADs re-distribute to nucleolar periphery (i.e. they become NADs).



From Pombo & Dillon, 2015

How many proteins ?

Moodle Activity 2

Effects of nuclear pore proteins on gene positioning

Multiple constituents of the NE contribute to genome organization. In addition to lamins, components of the nuclear pore complex (NPC) show chromatin association

Geyer et al., 2011

The nuclear envelope and transcriptional control

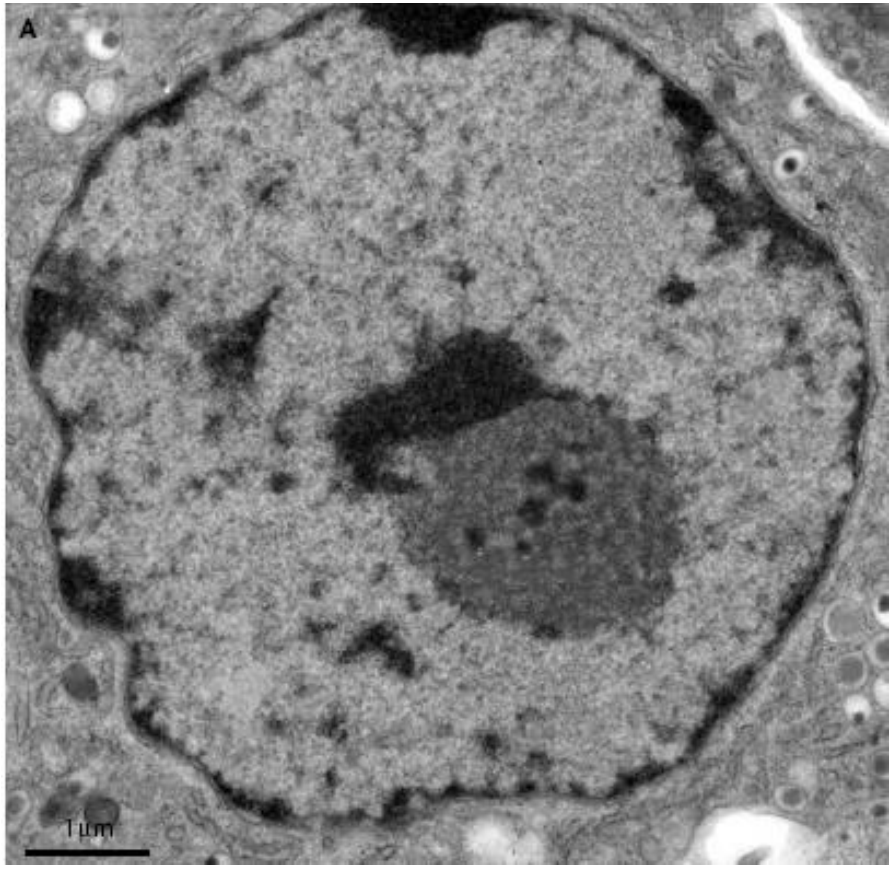
Asifa Akhtar and Susan M. Gasser‡*

Abstract | Cells have evolved sophisticated multi-protein complexes that can regulate gene activity at various steps of the transcription process. Recent advances highlight the role of nuclear positioning in the control of gene expression and have put nuclear envelope components at centre stage. On the inner face of the nuclear envelope, active genes localize to nuclear-pore structures whereas silent chromatin localizes to non-pore sites. Nuclear-pore components seem to not only recruit the RNA-processing and RNA-export machinery, but contribute a level of regulation that might enhance gene expression in a heritable manner.

2007

Akhtar & Gasser, 2007; Nat Rev Gen 8:507-517.

A



B

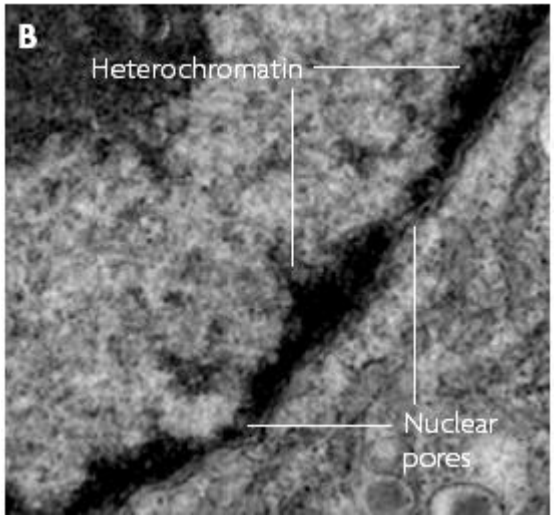


Figure 1 | **Heterochromatin in mammalian and yeast cells is distinct from nuclear pores. A** | An electron micrograph of the mammalian liver nucleus (with an enlarged section shown in part **B**), showing dense-staining heterochromatin located around the nucleolus and against the nuclear envelope. Nuclear pores open onto lighterstaining open chromatin.

Akhtar & Gasser, 2007; Nat Rev Gen 8:507-517.

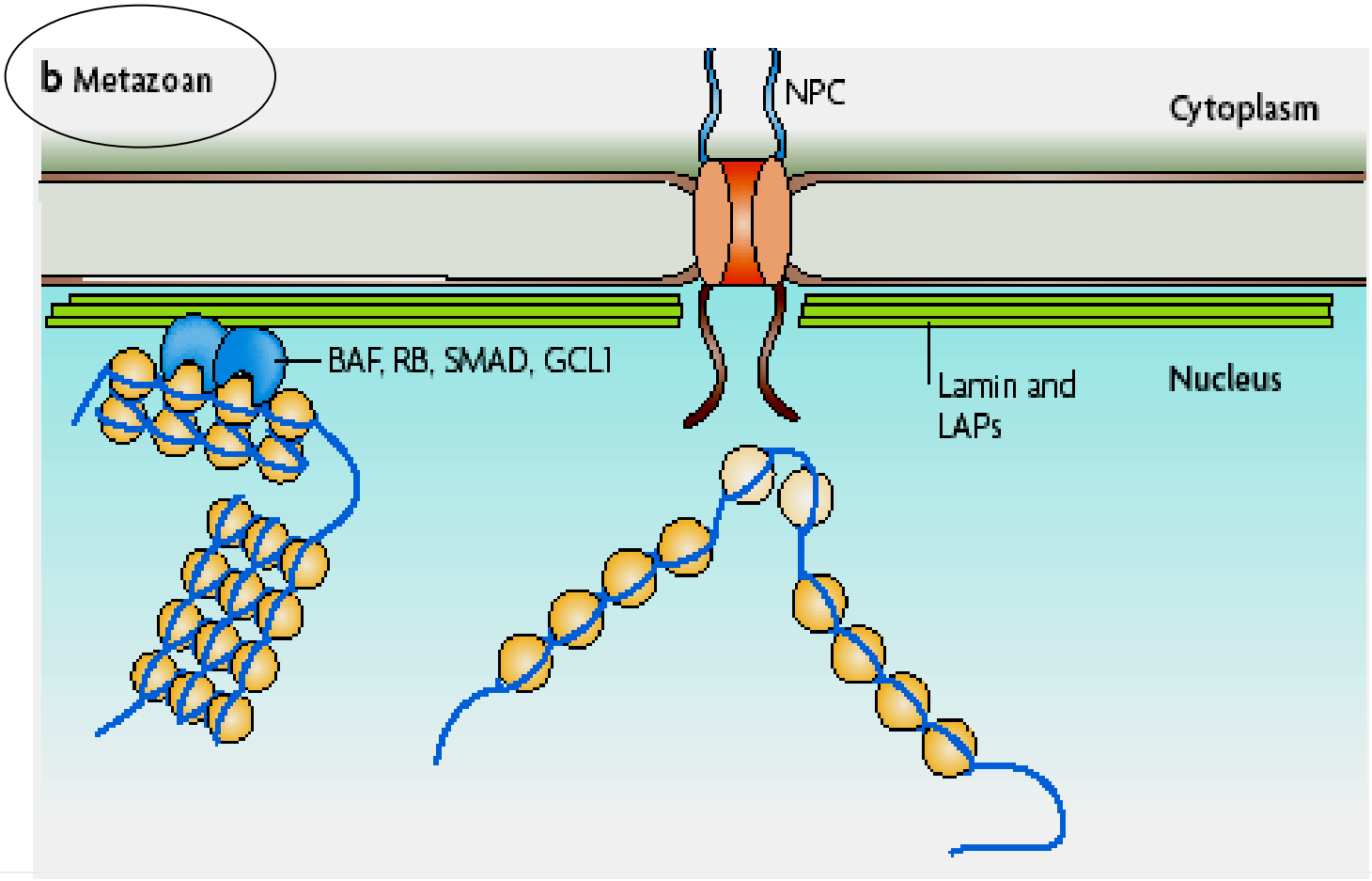
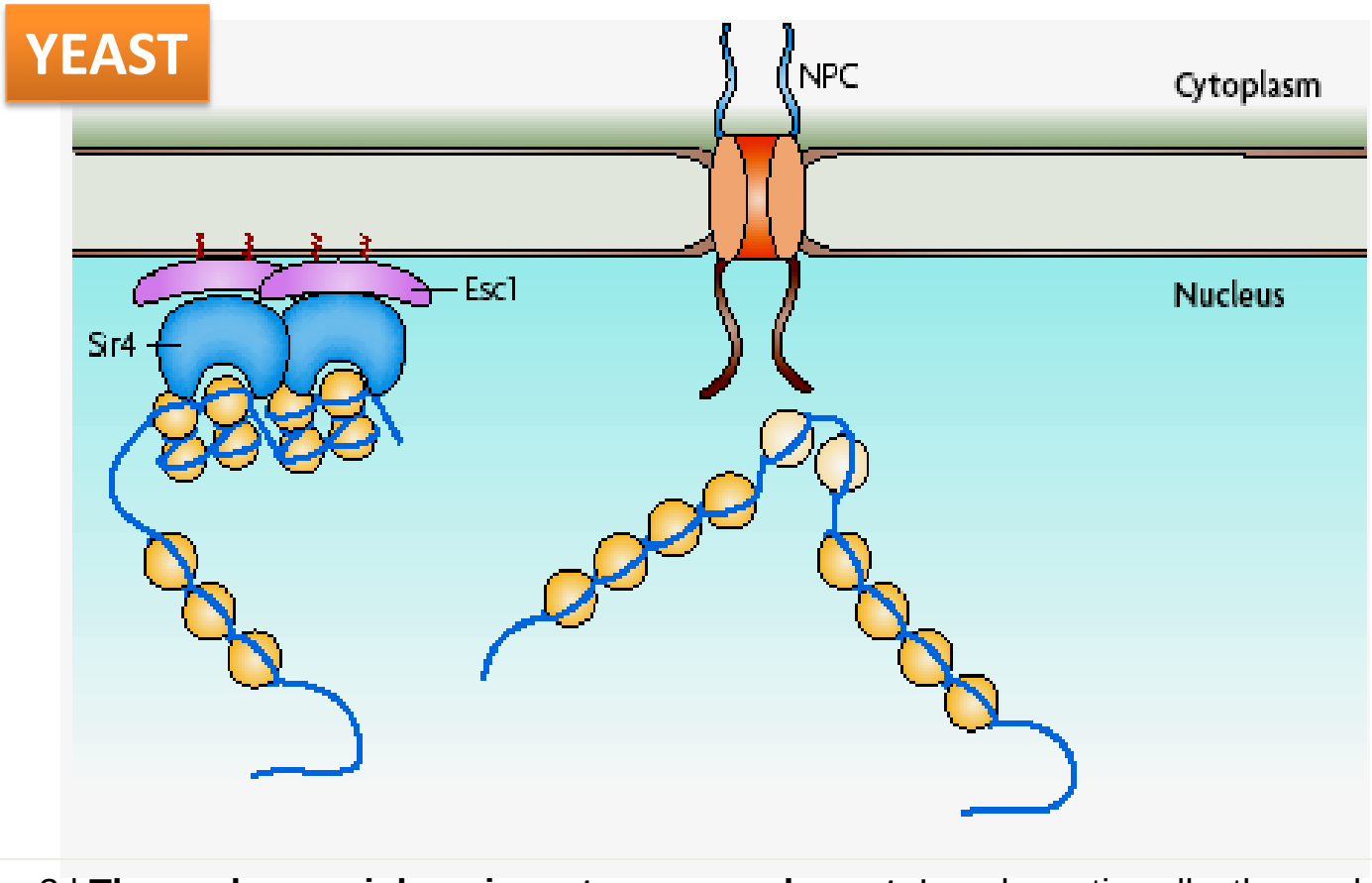


Figure 2 | **The nuclear periphery in metazoans and yeast.** In eukaryotic cells, the nuclear compartment is separated from the cytoplasm by the inner and outer nuclear membranes. This membrane bilayer is perforated by nuclear pores, which are constituted by a large multiprotein complex (the nuclear pore complex (NPC)) that is composed of about 30 proteins. This nuclear membrane, together with the pores, is commonly referred as the 'nuclear envelope' (NE).

b | In metazoan nuclei, the nuclear envelope is underlaid by a continuous meshwork of lamins and lamin-associated proteins (LAPs), which preferentially associate with inactive chromatin regions. Increasing evidence implicates interactions of chromatin with various nuclear-envelope components in gene repression as well as gene activation. BAF, barrier to autointegration factor; GCL1, germ-cell-less homologue; RB, retinoblastoma 1.



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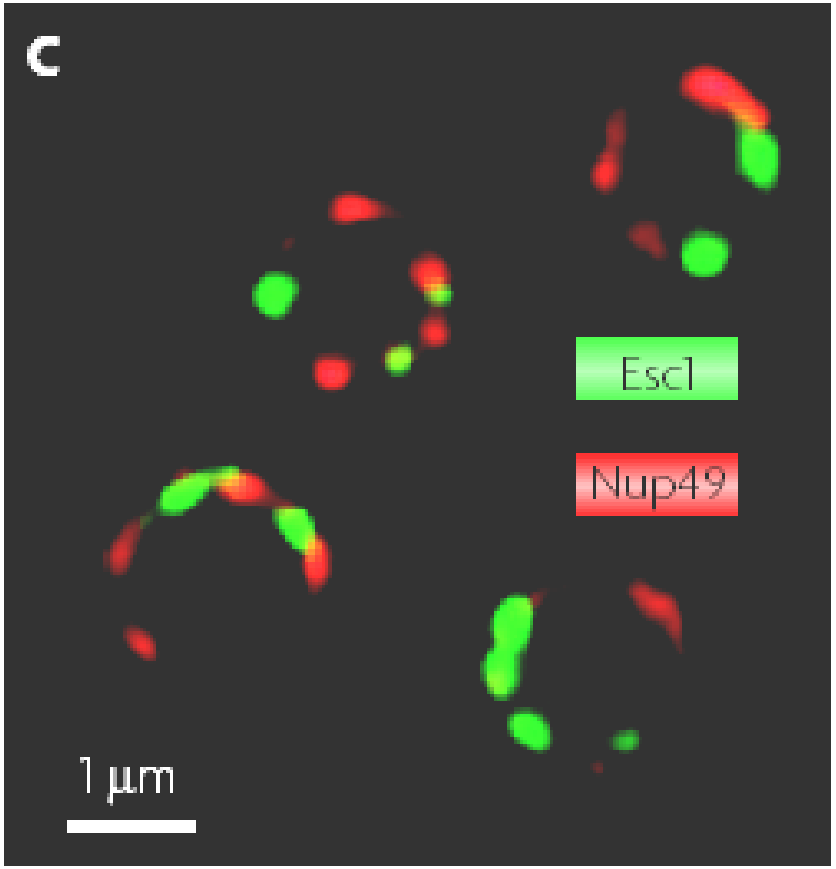
a | In **yeast** nuclei, envelope-associated proteins such as Esc1 (enhancer of silent chromatin 1) are present in foci at the periphery; however, they do not coincide with the pores. Esc1 binds Sir4 (silent information regulator 4), which is an integral component of repressed heterochromatin in yeast.

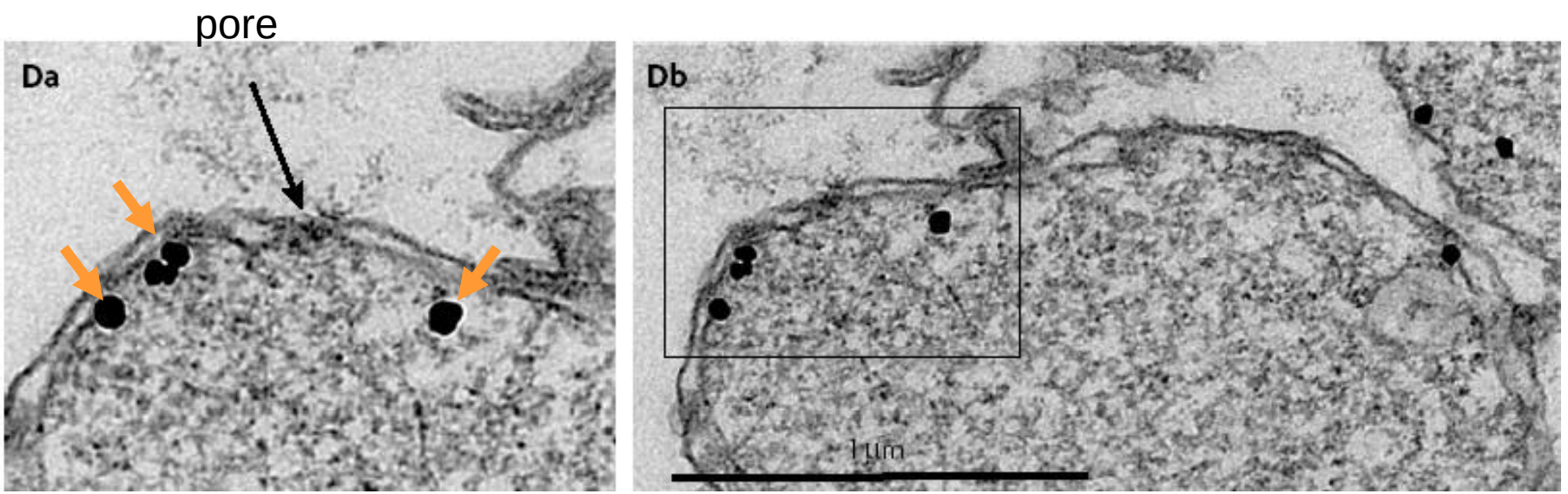
Akhtar & Gasser, 2007; Nat Rev Gen 8:507-517.

Yeast subtelomeric heterochromatin is made by hypoacetylated nucleosomes bound by a protein complex called «Silent information regulators» (Sir2, Sir3 and Sir4).
Sir4 interacts with the membrane associated Esc1 protein.



C | In budding yeast, heterochromatin binds the nuclear envelope through Esc1 (**enhancer of silent chromatin 1**; labelled green), which forms distinct foci alternating with nuclear pores (visualized in red through labelling of Nup49 (**nucleoporin 49**).

Akhtar & Gasser, 2007; Nat Rev Gen 8:507-517.



D | An electron micrograph showing **Esc1** at non-pore sites along the yeast inner nuclear envelope. An arrow indicates the nuclear pore, and black dots represent the labelling of **Myc-epitope-tagged Esc1** using fluoronanogold Alexa488 anti-mouse antibody18.

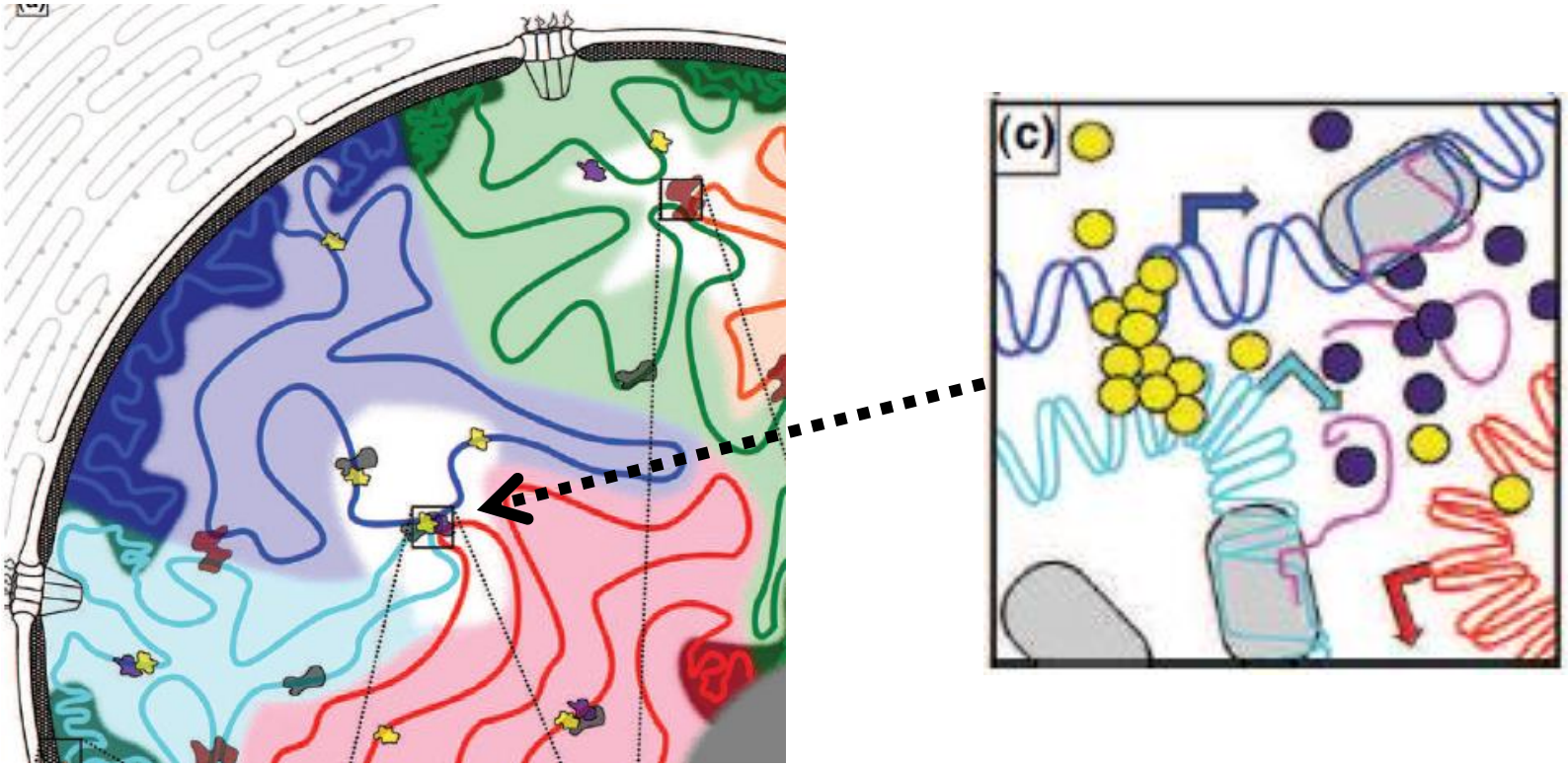
Akhtar & Gasser, 2007; Nat Rev Gen 8:507-517.

Contribution of transcriptional machinery to nuclear organization

The interchromosomal space located between CTs contains a variety of nuclear substructures, referred to by many names including foci, speckles, bodies and spots (Figure 1a). The number and composition of these bodies depends on cell type [25]. Nuclear bodies are enriched in specific factors, such as those involved in transcription and RNA processing. While the function of these bodies has been challenging to discern, recent studies demonstrate an important role in nuclear organization.

Foci – speckles – bodies - spots

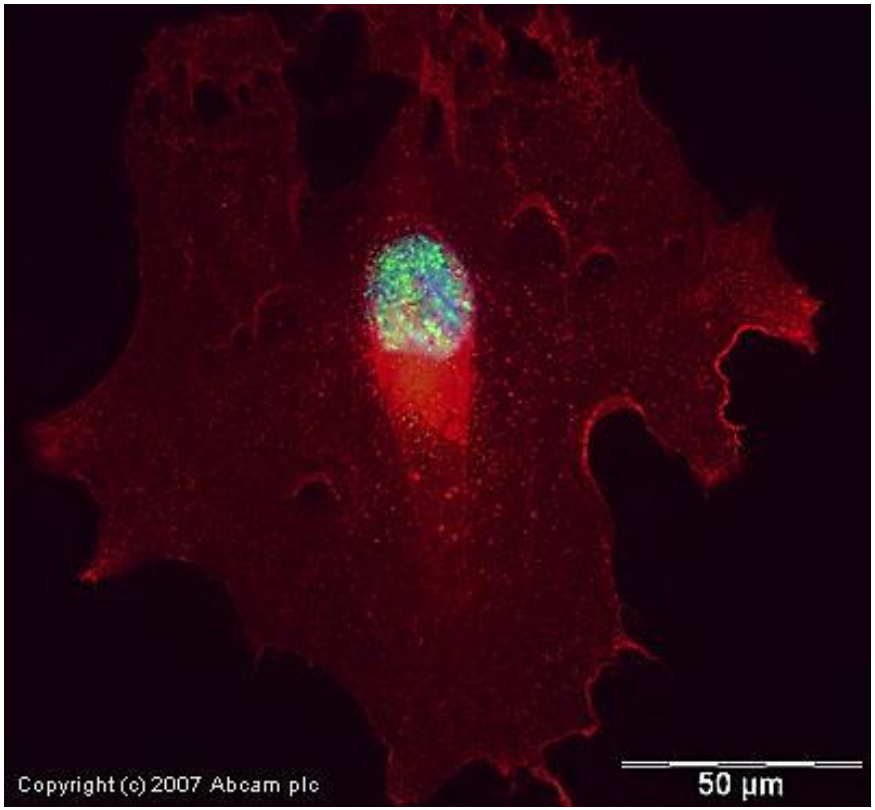
Geyer et al., 2011



Actively transcribing RNA Polymerase II exhibits a non-uniform distribution within interchromosomal spaces (Figure 1a). These 'transcription factories' have varying composition, with some enriched for RNA splicing factors, such as SC35 [26] (Figure 1c). Localization within

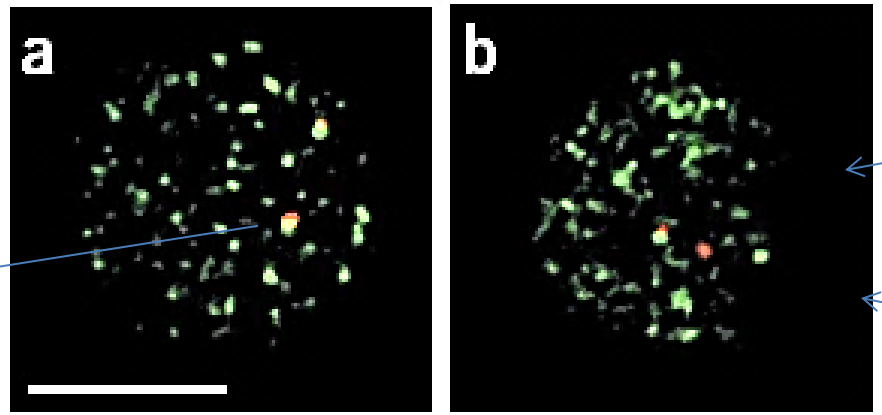
Geyer et al., 2011

Immunocytochemistry/ Immunofluorescence - RNA polymerase II CTD repeat YSPTSPS (phospho S5) antibody - ChIP Grade (ab5131)



Abcam website

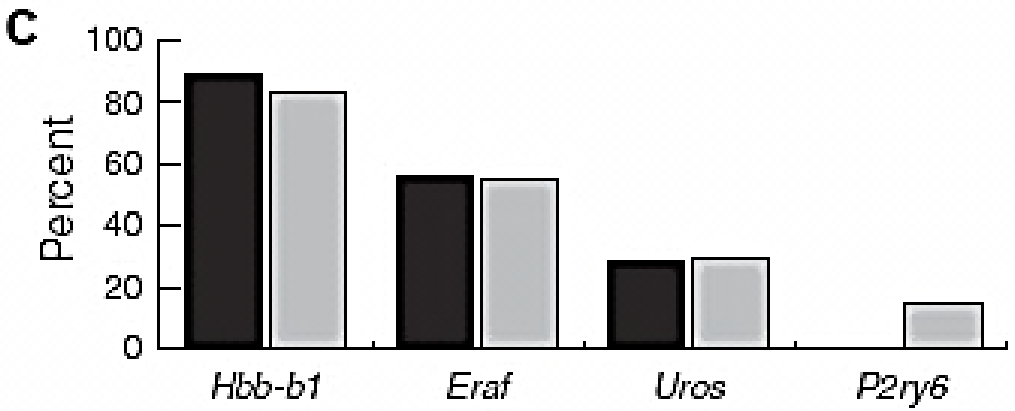
ICC/IF image of ab5131 stained human HeLa cells. The cells were methanol fixed (5 min), permabilised in TBS-T (20 min) and incubated with the antibody (ab5131, 1µg/ml) for 1h at room temperature. 1%BSA / 10% normal goat serum / 0.3M glycine was used to quench autofluorescence and block non-specific protein-protein interactions. The secondary antibody (green) was Alexa Fluor® 488 goat anti-rabbit IgG (H+L) used at a 1/1000 dilution for 1h. Alexa Fluor® 594 WGA was used to label plasma membranes (red). DAPI was used to stain the cell nuclei (blue).



green spots: RNA Pol II

Red spot: RNA immuno-FISH

Red spots: DNA immuno-FISH of an active gene (see legend)



RNA immuno-FISH

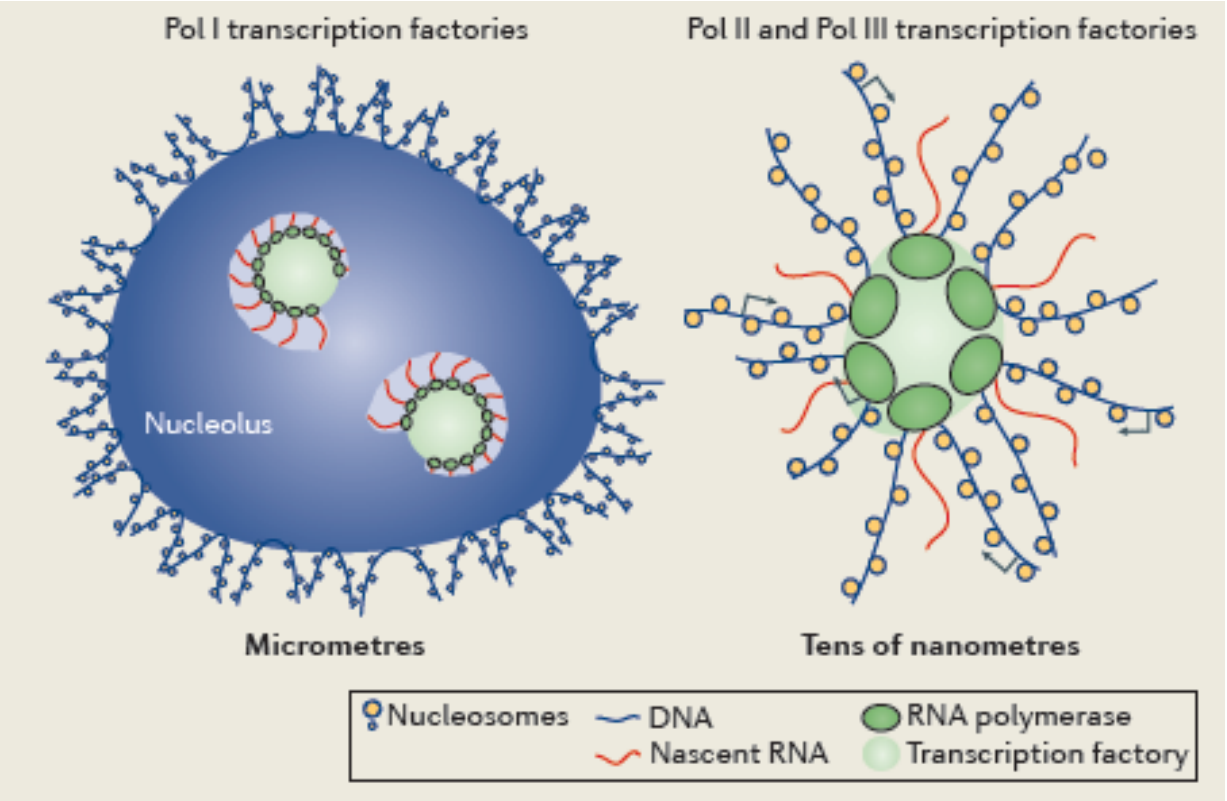
Figure 4 Actively transcribed genes associate with RNAP II foci. (a) RNA immuno-FISH of *Hbb-b1* transcription (red) with RNAP II staining (green) in anemic spleen erythroid cells. Scale bar, 5 μm. (b) DNA immuno-FISH of *Eraf* (red) with RNAP II staining (green). (c) Comparison of the percentage of alleles exhibiting a gene transcription signal by RNA FISH (black), with the percentage of loci that overlap with an RNAP II focus by DNA FISH (gray) for *Hbb-b1* ($n = 83$), *Eraf* ($n = 59$), *Uros* ($n = 47$) and *P2ry6* ($n = 79$).

Reference ?

Foci – speckles – bodies - spots

These supramolecular organizations (organelles) contain RNA Polymerases (many copies) and associated factors, including proteins involved in RNA processing (splicing factors, poly-Adenylation enzymes, capping enzymes etc.)

Transcriptional Factories



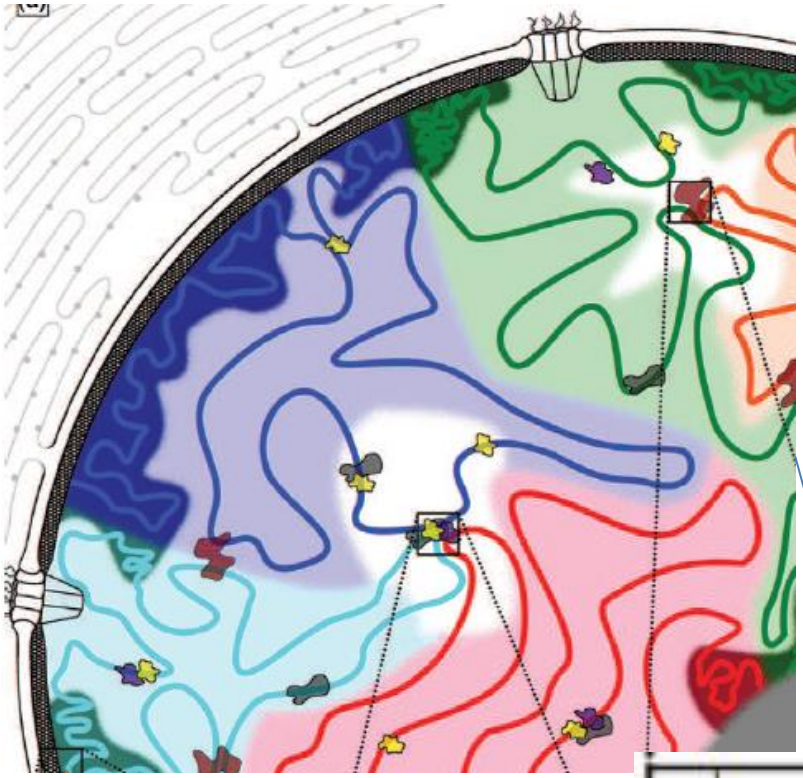
Pol I factories are found within **nucleoli** (see the figure, left), which in HeLa cells contain on average of 500 active enzymes and about four 45S rRNA genes, each of which is transcribed simultaneously by approximately 125 enzymes.
Individual nucleoli bring together 45S rRNA genes from clusters present in different chromosomes, constituting one of the first known examples of gene expression coordinated within a single structure

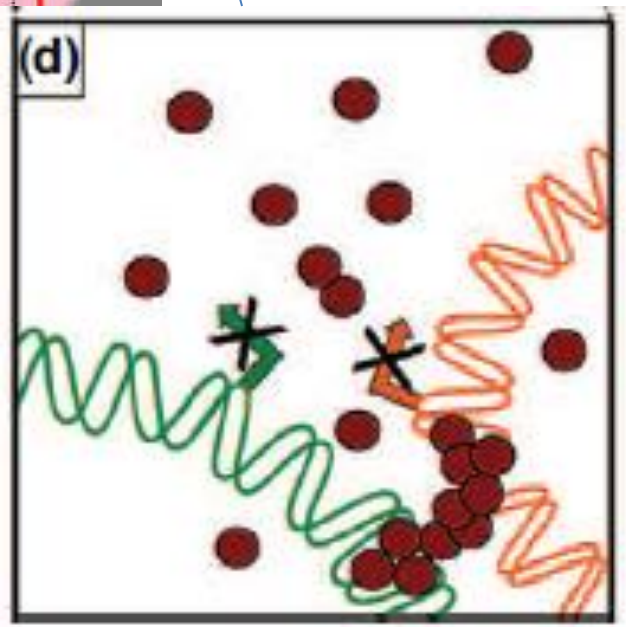
Pombo & Dillon 2015

Transcriptional repressors contribute to nuclear organization. This has been well documented for Polycomb group (PcG) proteins. PcGs play a role in the developmental repression of *Hox* gene clusters, which display an evolutionarily conserved chromosome arrangement and span megabases of DNA. Extensive studies of *Hox* genes in Drosophila demonstrated that transcriptional repression depends upon PcG proteins that associate with Polycomb Response Elements (PREs) distributed throughout the gene clusters* [30]. Repression correlates with coalescence of PREs into nuclear foci, termed Polycomb bodies that are present in the nuclei of most, but not all, tissues (Figure 1d) [31]. Polycomb bodies represent a

* In Mammals no PRE have been identified so far

Geyer et al., 2011



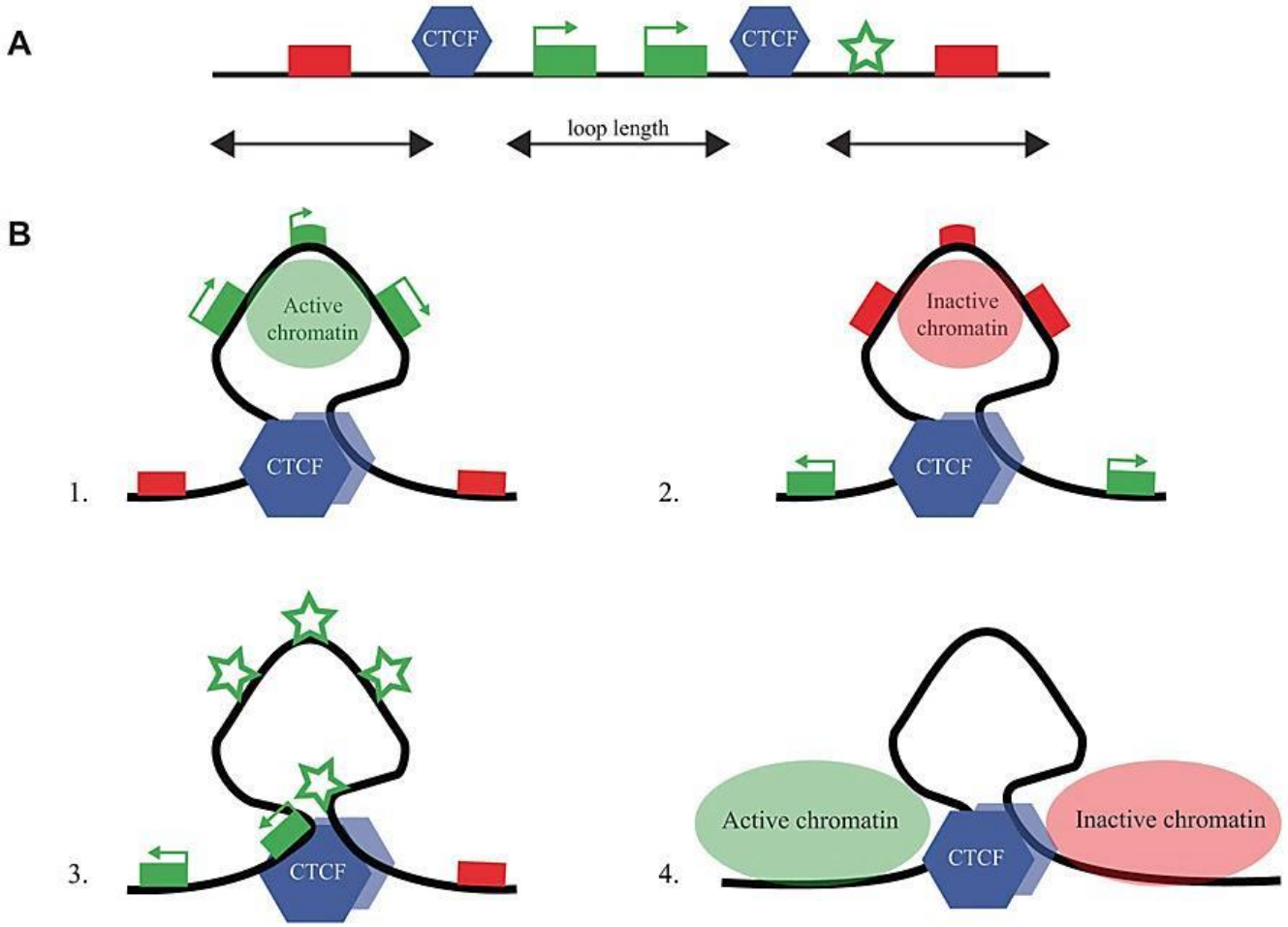


Polycomb bodies
Transcriptional Repressors

Insulator proteins also contribute to nuclear organization by establishing long-range and inter-chromosomal associations [35]. The insulator binding protein CCCTC binding factor (CTCF) is a multiple zinc finger protein with a highly conserved DNA binding domain.

CTCF participates in inter-chromosomal and intra-chromosomal interactions that regulate transcription. Interactions between CTCF and partner proteins such as cohesin and lamins might be crucial for positioning genes into subnuclear compartments.

Geyer et al., 2011

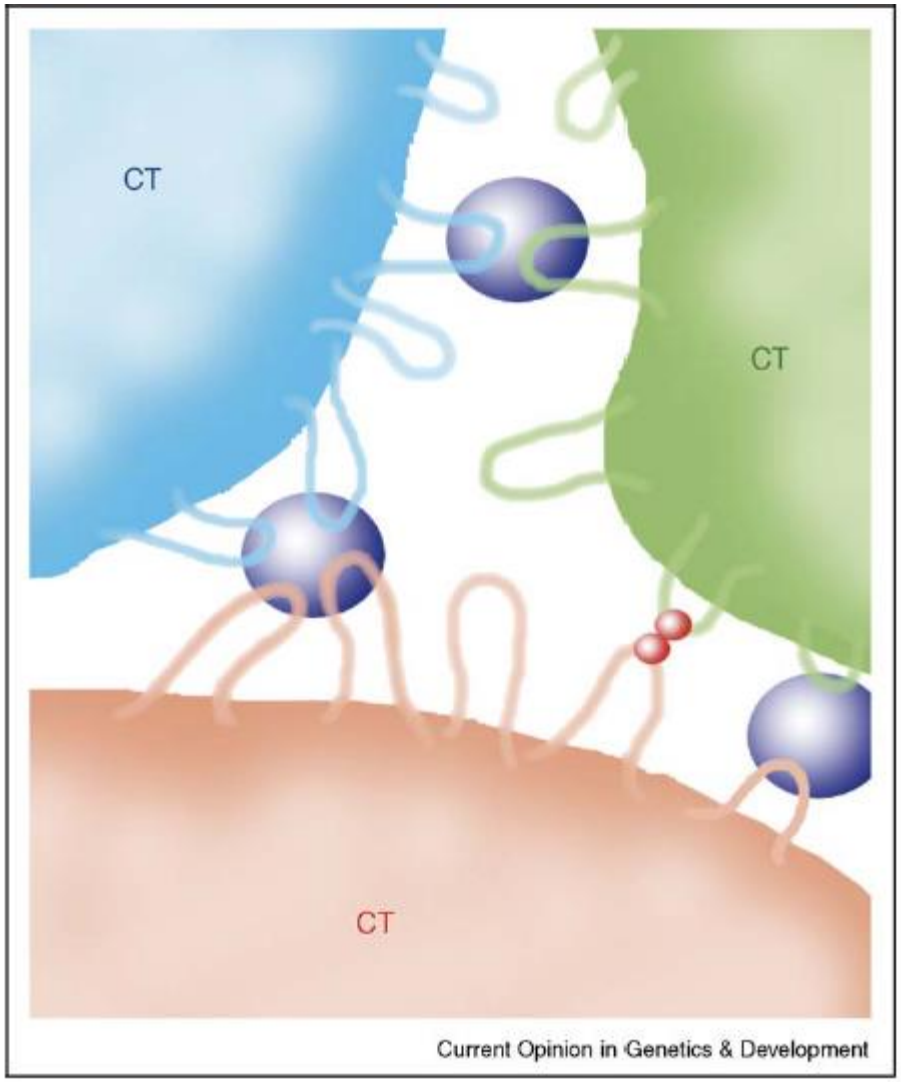


REVIEW ARTICLE

Front. Genet., 17 October 2012 | doi: 10.3389/fgene.2012.00217

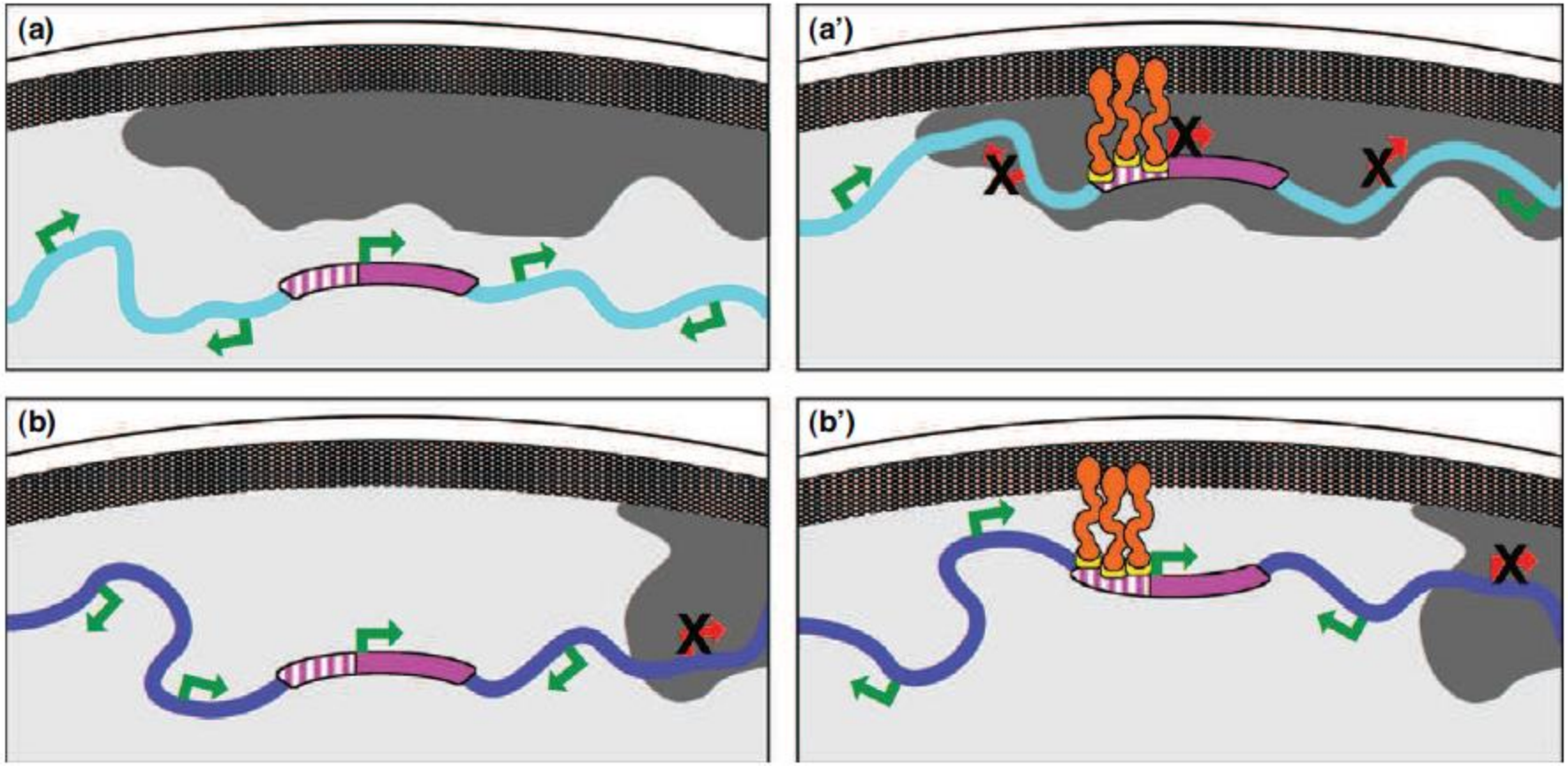
Chromatin loops, gene positioning, and gene expression

Sjoerd Holwerda and Wouter de Laat*



Nuclear organization. Three different chromosome territories (CTs) are shown. Potentially active genes in *cis* and *trans* dynamically engage transcription factories (blue spheres). Most 'active' genes spend the majority of their time outside transcription factories and are transcriptionally inactive. Other types of regulatory interactions (red spheres) might possibly sequester genes or inhibit mobility and factory contact.

Is differential localization of chromosomes (genes) cause or consequence ?



Geyer et al., 2011

Vol 452 | 13 March 2008 | doi:10.1038/nature06727

Research paper

Transcriptional repression mediated by repositioning of genes to the nuclear lamina

K. L. Reddy[1,2], J. M. Zullo[1,2], E. Bertolino[2] & H. Singh[1,2]

Nuclear compartmentalization seems to have an important role in regulating metazoan genes[1,2]. Although studies on immunoglobulin and other loci have shown a correlation between positioning at the nuclear lamina and gene repression, the functional consequences of this compartmentalization remain untested[2,3]. We devised an approach for inducible tethering of genes to the inner nuclear membrane (INM), and tested the consequences of such repositioning on gene activity in mouse fibroblasts. Here, using three-dimensional DNA-immunoFISH, we demonstrate repositioning of chromosomal regions to the nuclear lamina that is dependent on breakdown and reformation of the nuclear envelope during mitosis. Moreover, tethering leads to the accumulation of lamin and INM proteins, but not to association with pericentromeric heterochromatin or nuclear pore complexes. Recruitment of genes to the INM can result in their transcriptional repression. Finally, we use targeted adenine methylation (DamID) to show that, as is the case for our model system, inactive immunoglobulin loci at the nuclear periphery are contacted by INM and lamina proteins. We propose that these molecular interactions may be used to compartmentalize and to limit the accessibility of immunoglobulin loci to transcription and recombination factors.

In mammalian nuclei, chromatin is organized into structural domains by association with distinct nuclear compartments[2]. Several studies have shown a correlation between the transcriptional repression of mammalian genes and their positioning at the nuclear periphery[3–7]. In yeast, the nuclear periphery is comprised of at least two sub-compartments: a repressive compartment consisting of foci of silencing factors, and a permissive compartment involving nuclear pore complexes (NPCs) that facilitates gene expression[8–10]. However, metazoan systems exhibit a greater complexity of nuclear compartments and chromosome organization. The nuclear periphery in mammalian cells is constituted by a distinct set of INM proteins, such as LBR, LAP2 and emerin (EMD), as well as an underlying nuclear lamina, which have been proposed to interact with transcriptional repressors[11–14]. The ability of this nuclear compartment to regulate gene activity has not been functionally tested in metazoan cells[2].

We designed a two-component inducible system that would relocalize an integrated reporter gene from the interior of a mammalian nucleus to the INM (Fig. 1a). The reporter construct is comprised of the herpes simplex virus thymidine kinase promoter and the hygromycin resistance gene (*Tk-hyg*) as well as a nearby array of Lac operators (*lacO*) that constitute binding sites for the *Escherichia coli* Lac repressor (LacI) (Fig. 1 and Supplementary Fig. 1a)[15]. The second component is either a nucleoplasmic green fluorescent protein (GFP)–LacI that binds *lacO* sites and enables visualization of the reporter gene or a tethering protein GFP–LacI–ΔEMD that is targeted to the INM by means of a carboxy-terminal segment of EMD[16]. The GFP fusion proteins were stably expressed in NIH3T3 fibroblast clones harbouring the reporter gene(s) integrated at single (S) or multiple (M) chromosomal sites. Reporter gene visualization and/or repositioning were controlled using the allosteric inhibitor IPTG (isopropyl β-D-1-thiogalactopyranoside), which regulates LacI binding to *lacO* sites. The initial disposition of the integrated reporter genes was analysed in cells stably expressing GFP–LacI. Up to four bright GFP foci were visible in clone-M nuclei because these cells have four integration sites, each containing multiple copies of the reporter gene (Supplementary Fig. 1d, e). In contrast, clone-S nuclei exhibited dimmer single GFP foci owing to a single site of insertion with fewer copies (1–2) of the reporter (Supplementary Fig. 1c, d). We next generated clone-M and clone-S derivatives stably expressing GFP–LacI–ΔEMD. As anticipated, this tethering protein localized to the INM. On removal of IPTG, large GFP foci were observed at the nuclear periphery in clone-M but not in clone-S cells expressing GFP–LacI–ΔEMD (Supplementary Fig. 1e). This suggested that the reporter genes were being repositioned to the nuclear membrane in clone-M cells.

Not all tethered reporter genes were expected to accumulate the fusion protein at levels that are discernable as fluorescent signals above the distribution in the INM. This was probably the case for clone-S cells. Therefore, we undertook fluorescent DNA *in situ* hybridization on three-dimensional preserved nuclei (3D DNA-immunoFISH) to assess quantitatively the disposition of all *Tk-hyg* integrations (Fig. 1b, c and Supplementary Fig. 2). Under control conditions, the integrated reporter genes were distributed throughout the nucleoplasm, with approximately 25–30% being positioned near the nuclear periphery (Fig. 1d). This frequency represents the initial sub-nuclear distribution and is similar to that observed for endogenous genes that are not associated with the nuclear periphery[17]. On withdrawal of IPTG, most *Tk-hyg* insertions were found to be associated with the nuclear lamina in clone-M (70%) and clone-S (90%) cells expressing GFP–LacI–ΔEMD. Moreover, in clone-M cells, reporter genes residing on different chromosomes were repositioned to distinct regions of the INM in a single nucleus (Fig. 1b). In clone-S cells, repositioning was mediated by fewer copies of the *lacO* segments (1–2) compared with in clone-M cells (~25 copies per integration site, Supplementary Fig. 1d). We note that repositioning requires breakdown and reformation of the nuclear envelope during mitosis (Supplementary Fig. 3 and Supplementary Discussion). These data provide the first demonstration of directed repositioning of chromosomal segments to the INM–lamina compartment, and suggest that an intervening cell cycle may be necessary for such re-configuration.

We next analysed the consequences of accumulating GFP–LacI–ΔEMD at sites of tethering on the disposition of other proteins at the INM. Lamin A and B1, key components of the lamina, and the INM protein LAP2 accumulated at sites of tethering (Fig. 2a and Supplementary Fig. 4). No such interactions were observed on non-tethered

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Technical

- Dam-ID
- Chromatin Immunoprecipitation
- 3C – Chromosome Conformation Capture

One (or two) single loci

versus

Genome-wide

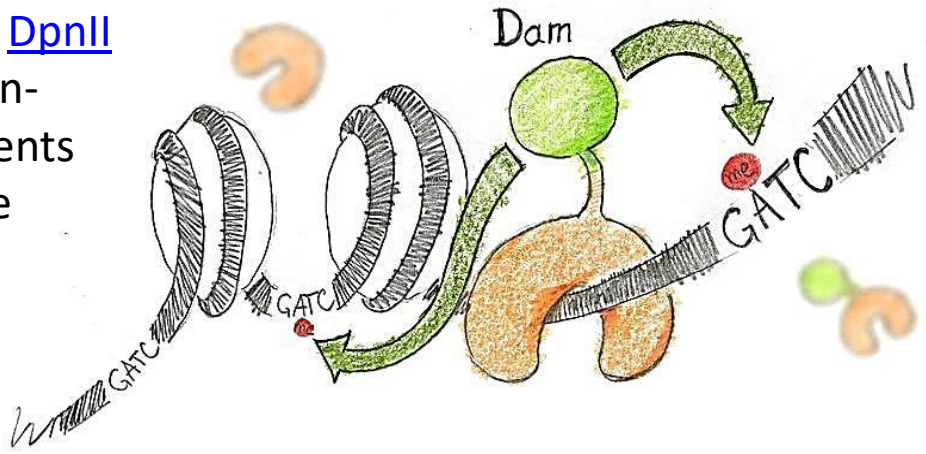
Dam ID

N6-methyladenine (m6A) is the product of the addition of a methyl group (CH_3) at position 6 of the adenine. This modified nucleotide is absent from the vast majority of eukaryotes.

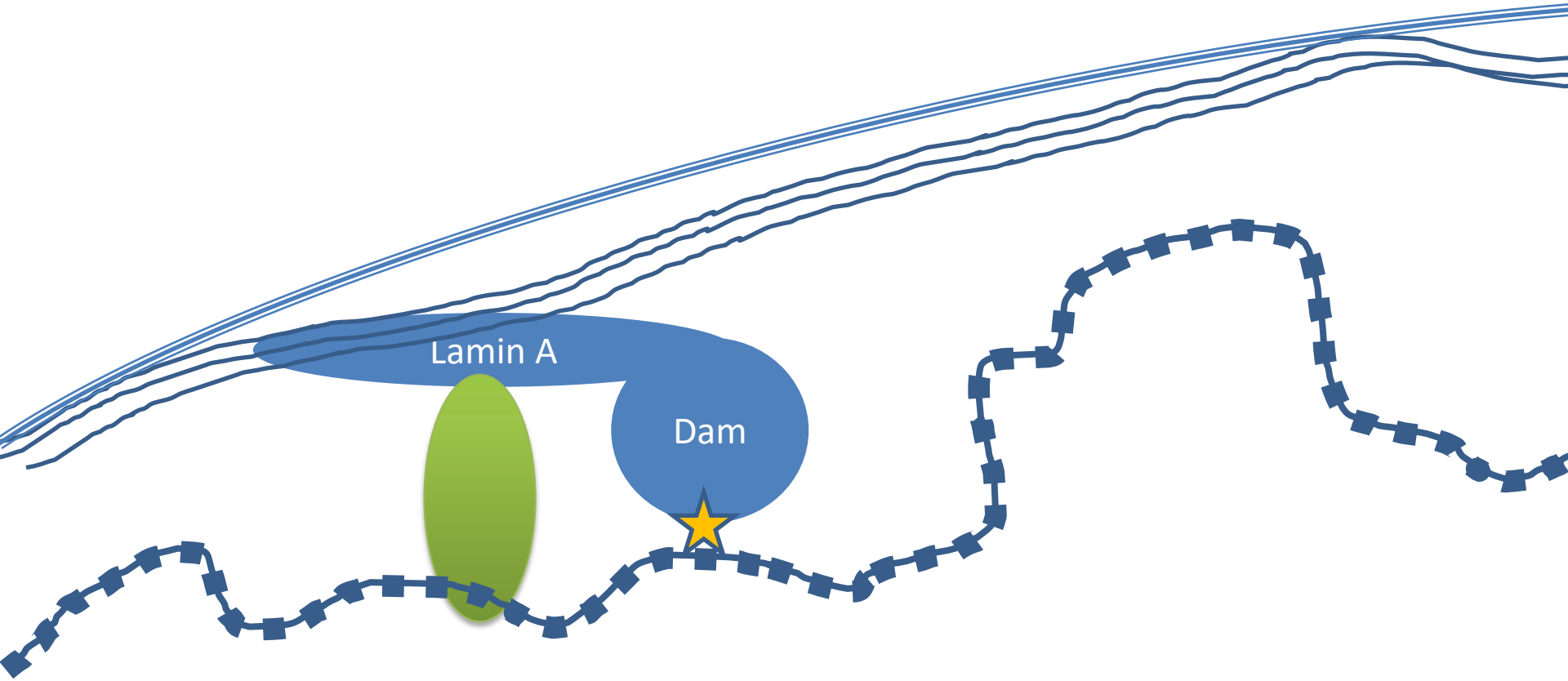
Dam-ID is a proximity assay

Methyl PCR (mePCR)

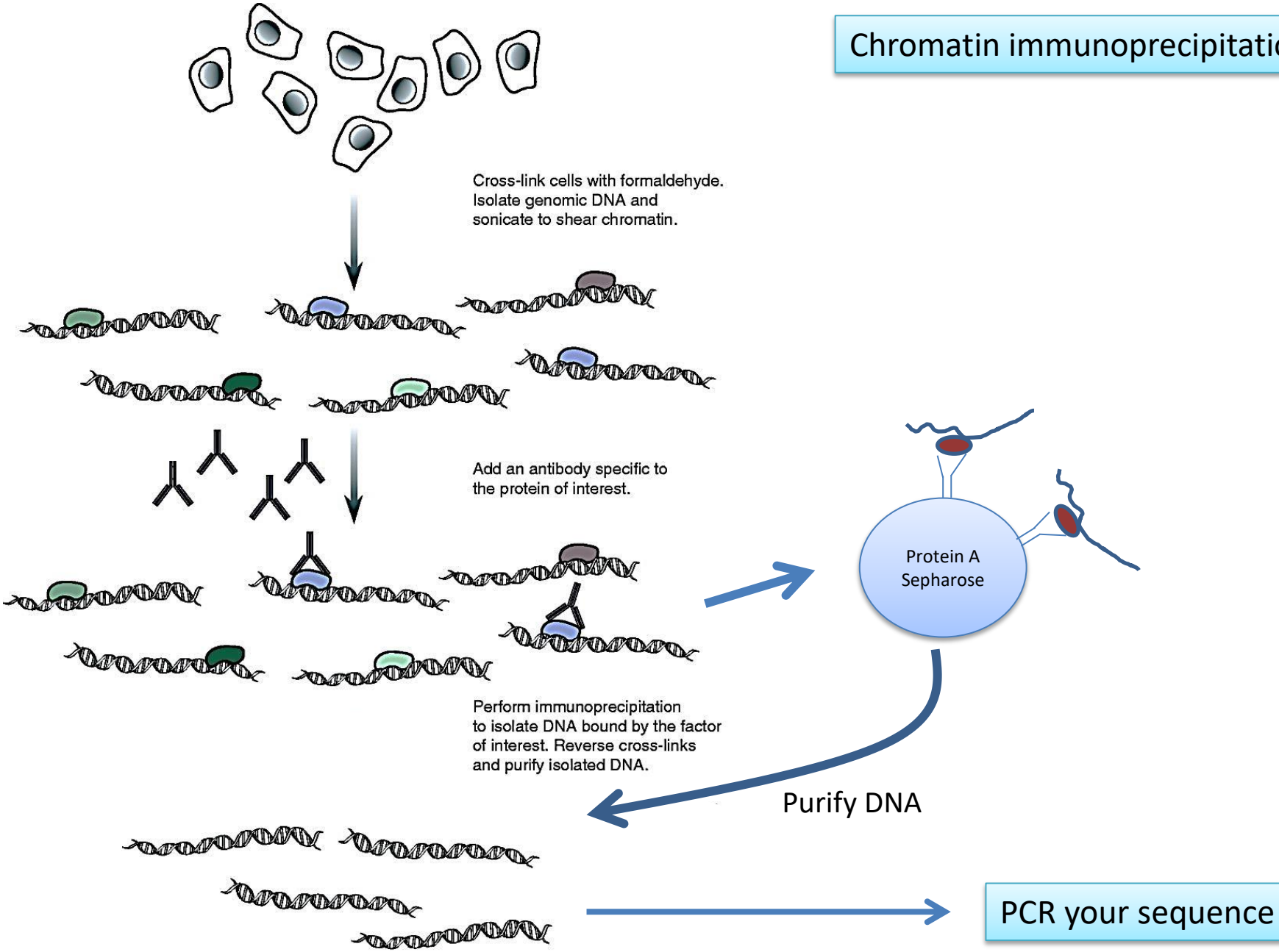
In this assay the genome is digested by DpnI, which cuts only methylated GATCs. Double-stranded adapters with a known sequence are then ligated to the ends generated by DpnI. A PCR with primers matching the adaptors is then carried out, leading to the specific amplification of genomic fragments flanked by methylated GATCs. In practice, ligation products are digested by DpnII prior PCR amplification. This enzyme cuts non-methylated GATCs, ensuring that only fragments flanked by *consecutive* methylated GATCs are amplified.



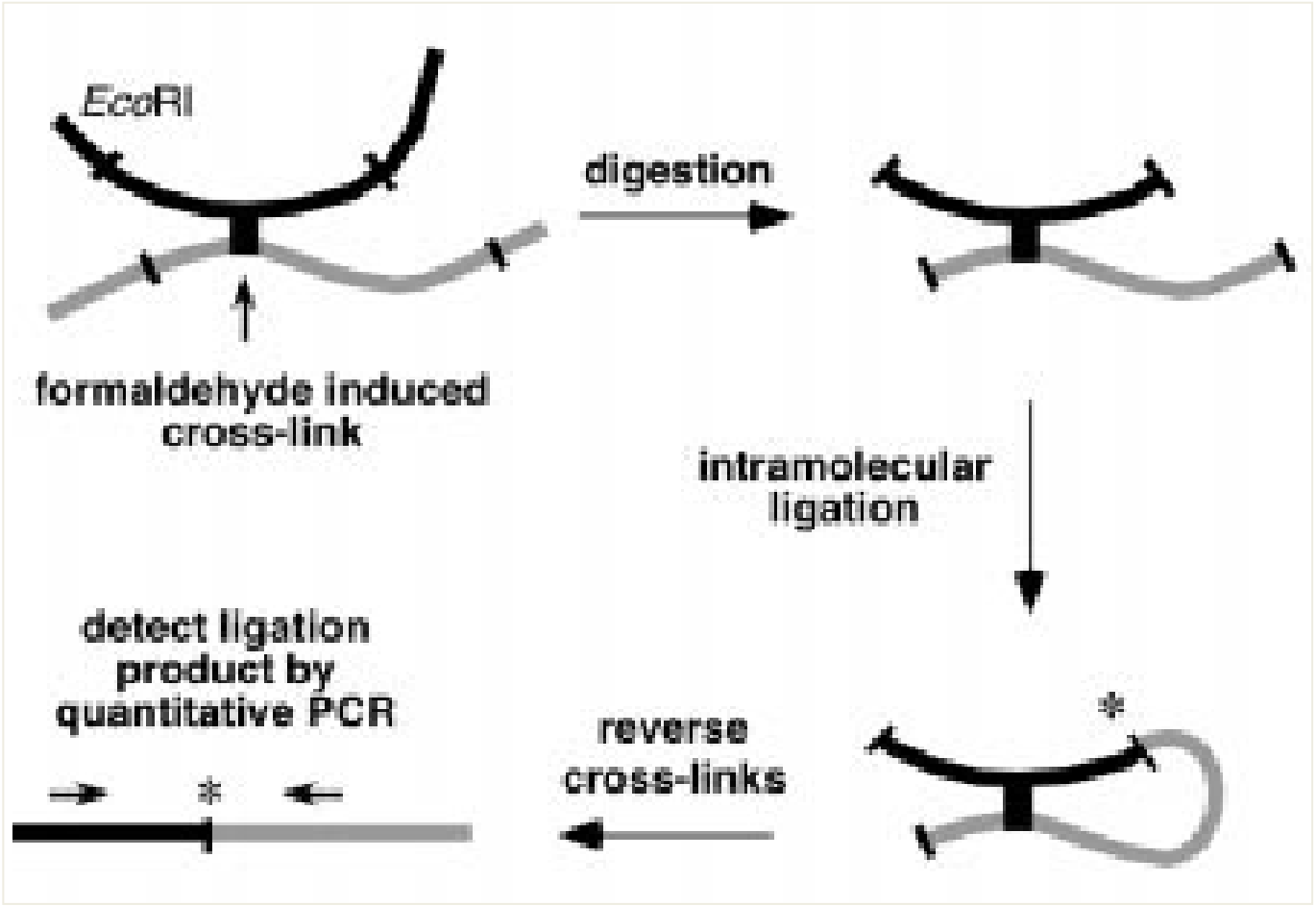
This figure refers to a DNA-binding protein


Lamin A
Dam

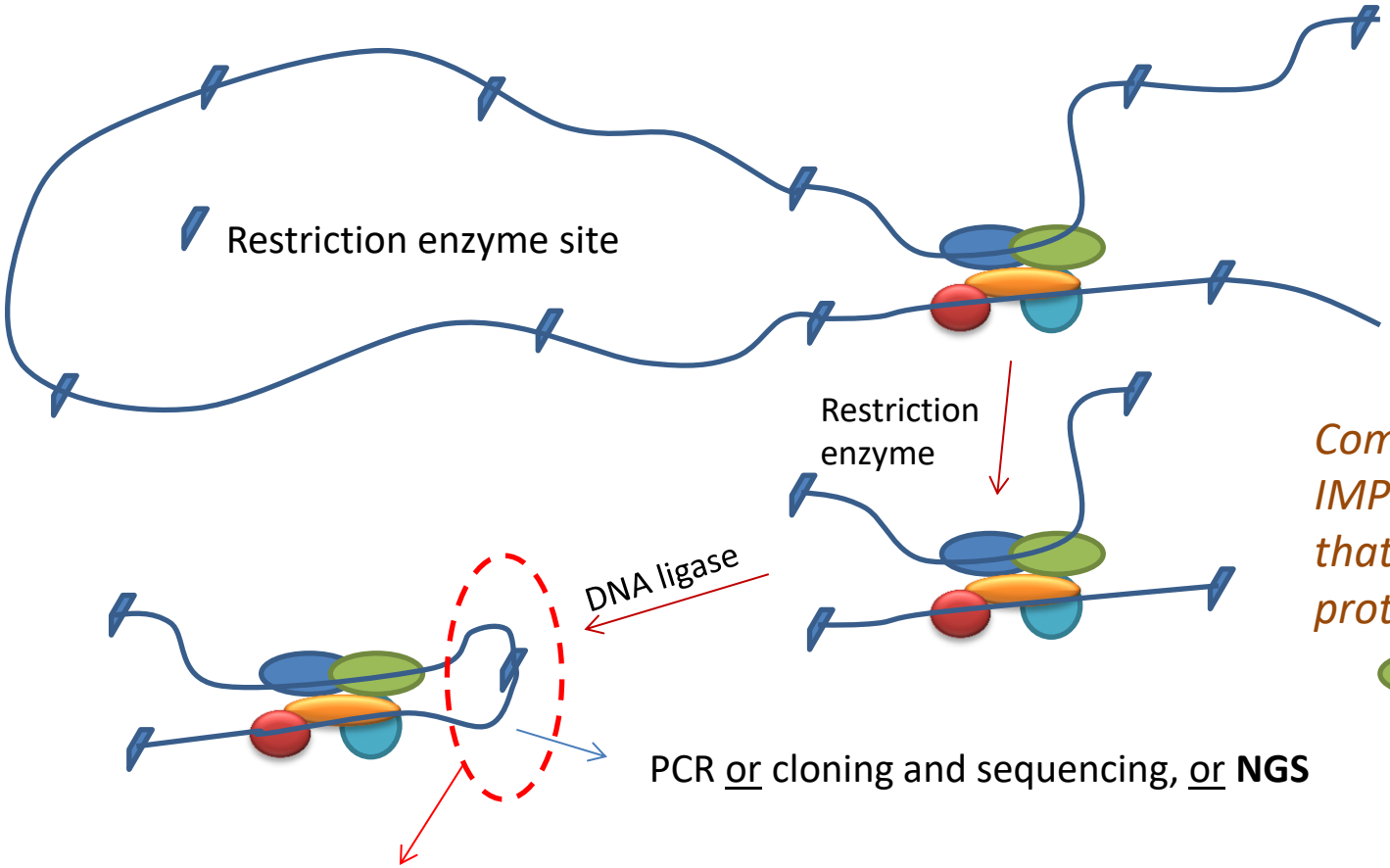
Chromatin immunoprecipitation



The basic methodology to study Enhancer-Promoter interaction is 3C assay = chromosome conformation capture



Long-range interactions are studied with 3C (Chromatin Conformation Capture) or different genome-wide scale variants (4C, 5C, Hi-C, ChIA-PET).



Complexes may also be IMPT using an antibody that recognizes a specific protein → ChIA-PET

PCR for single interaction.
Generate libraries to NGS for genome-wide studies

Note: from this scheme nucleosomes are omitted

NGS

Fragment the DNA (or RNA) to be sequenced in smaller pieces

Physically separate the fragments

High-parallel sequencing of fragments

No cloning step required

Next Generation Sequencing

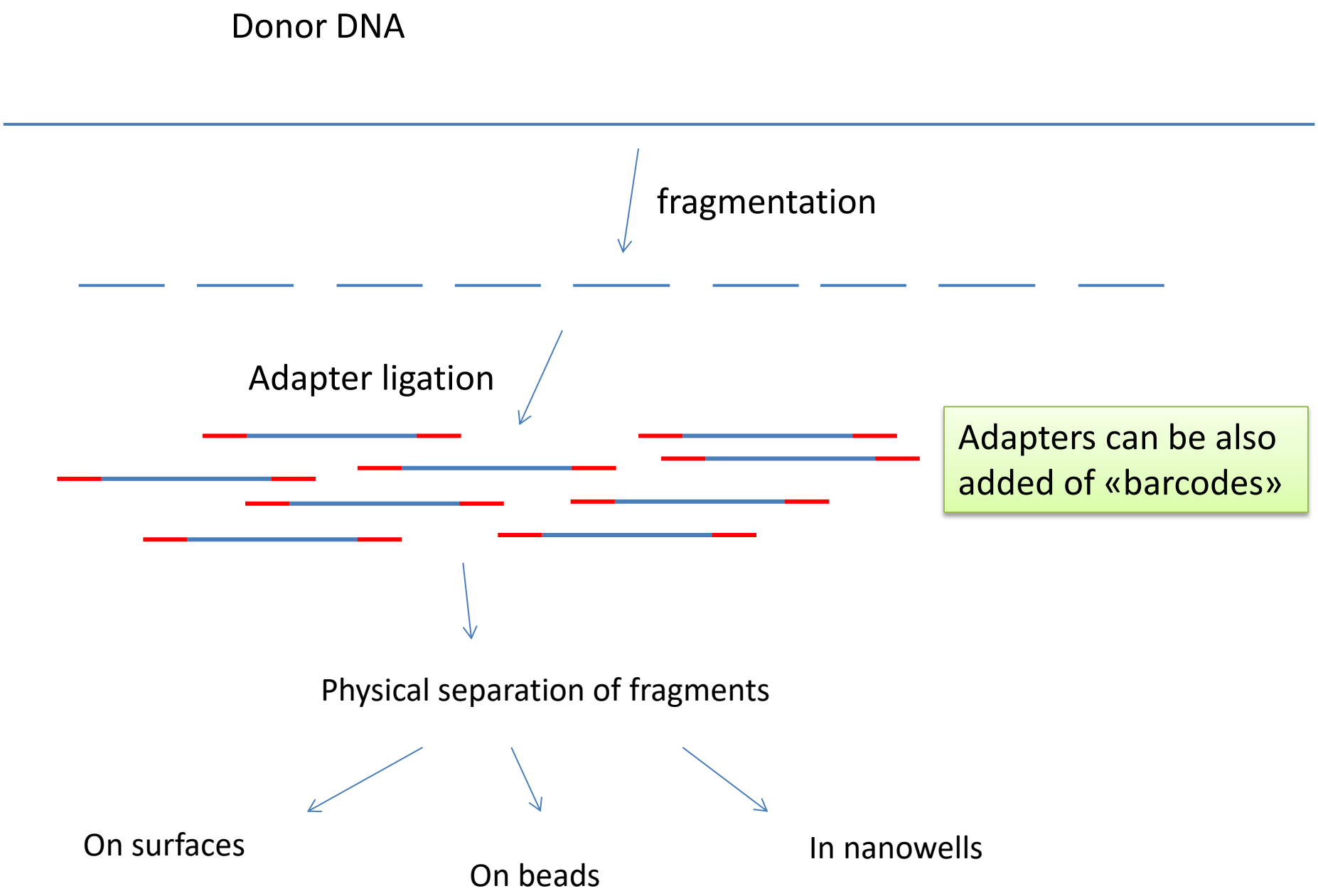
(deep-sequencing / mass sequencing)

✓generation of “DNA-nanoclones” on distinct solid surfaces by PCR or single-molecule isolation

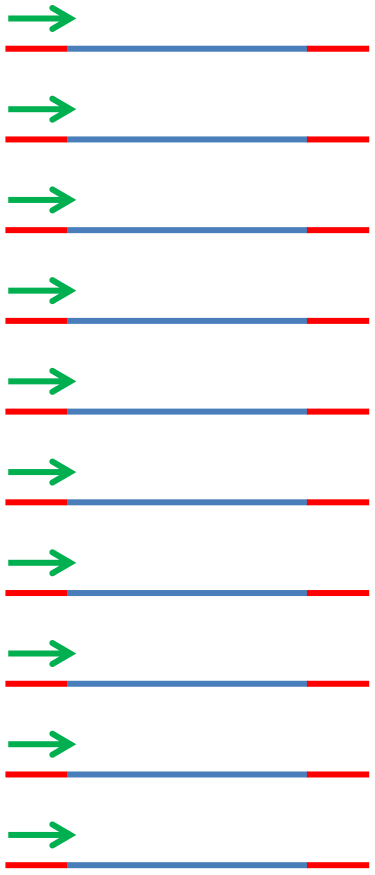
✓highly parallel in situ sequencing

✓record read-out i.e. millions or short sequences (“**reads**”)

✓align reads on genomes or assembly

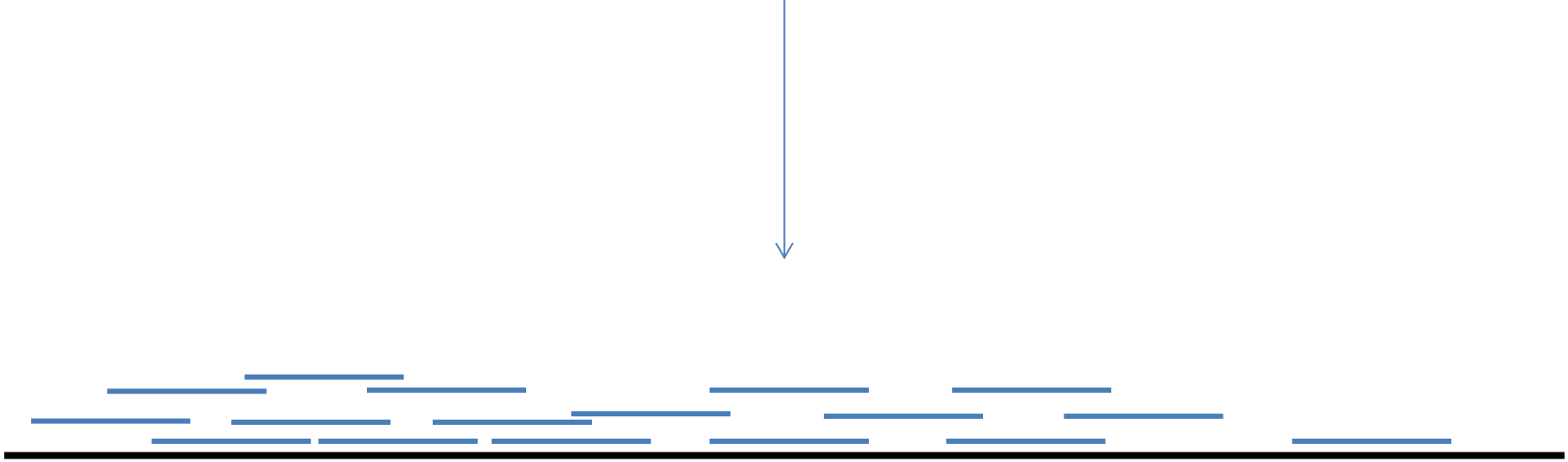

Donor DNA
fragmentation
Adapter ligation
Adapters can be also added of «barcodes»
Physical separation of fragments
On surfaces
On beads
In nanowells

Adapter-primed sequencing by synthesis in high-parallel mode



Millions of sequence reads
In parallel

Reads are mapped to the reference genome



reference genome

In NGS sequencing, the number of independent sequences (called «reads») is more important than lenght

The % of reference genome that is represented in «reads» is the «**coverage**».

Other essential aspects:

1) speed
2) cost
3) error-to-depth ratio

Next generation sequencing methods:

Number of molecules per sequence

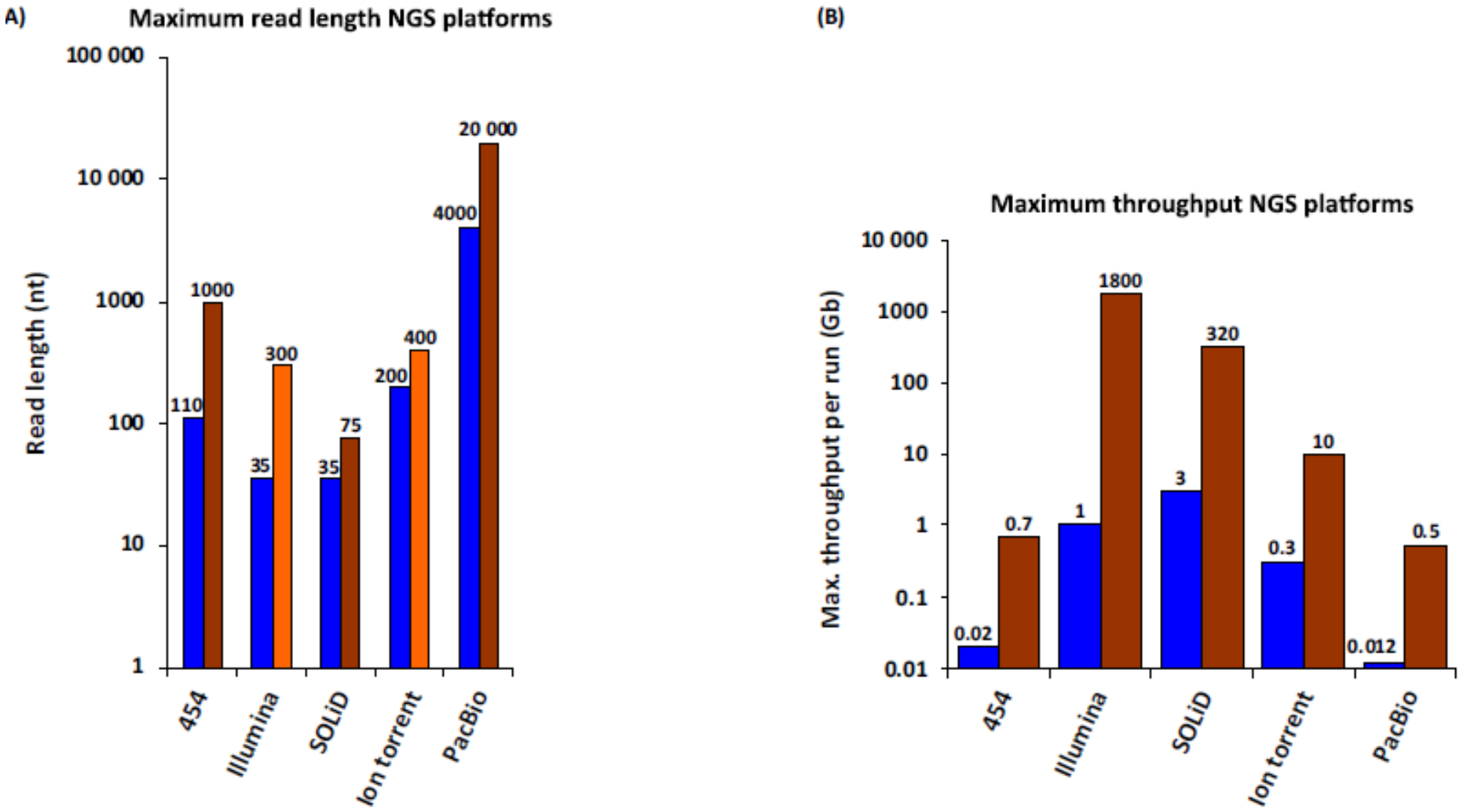
- Amplification
- Single-molecule

Biochemical measurement

- Sequencing by synthesis (Sanger is synthesis + termination)
  - Nucleotide chemistry
  - Associated chemistry
- Sequencing by annealing and ligation
- Sequencing by direct physico-chemical measurements

Detection

- Optical detection
- Ion or conductance detection



In blue the first version of the instruments

From Van Dijk et al., 2014 (Textbook)