L1.4

Research Paper 1

Discussion

L0 – Introduction to Regulatory Genomics

L1.1 – Nuclear Organization

L1.2 – Nucleus topography

L1.3 – Chromatin structures & composition

L1.4 (today) - Research Paper 1

L2.1 - Genomes

L2.2 - Epigenomes

L2.3 – Epigenetic inheritance

Repositioning to nuclear lamina drives repression

Research paper

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

LETTERS

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]

Letters are quicker reports that Articles

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

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

The Authors: first and last have major responsibility

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PubMed citation

Reddy KL, Zullo JM, Bertolino E, Singh H. Transcriptional repression mediated by repositioning of genes to the nuclear lamina. Nature. 2008 Mar13;452(7184):243-7. doi: 10.1038/nature06727. Epub 2008 Feb 13. PubMed PMID:18272965.

Full citation

Reddy KL, Zullo JM, Bertolino E, Singh H. Transcriptional repression mediated by repositioning of genes to the nuclear lamina (2008). Nature 452:243-247.

Short citation

Reddy et al., Nature 452:243-7 (2008)

Nature (and othe scientific journals like Science, for example) have different formats for papers.
Articles report complete research work
Letters are quicker reports than Articles
News & views are short reviews on a recently reported research

Nature Letters do not have the classical structure of Introduction, Materials & Methods, Results, Discussion as other journals do.

But they do…

Abstract

introduction

purpose

results

conclusions

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

Introduction

globulin 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].

Specific question

We designed a two-component inducible system that would relo-

Results start here

Methodology, i.e. The experimental approach invented by the Authors to investigate the specific issue outlined

Fig. 1a

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

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Supplementary ???

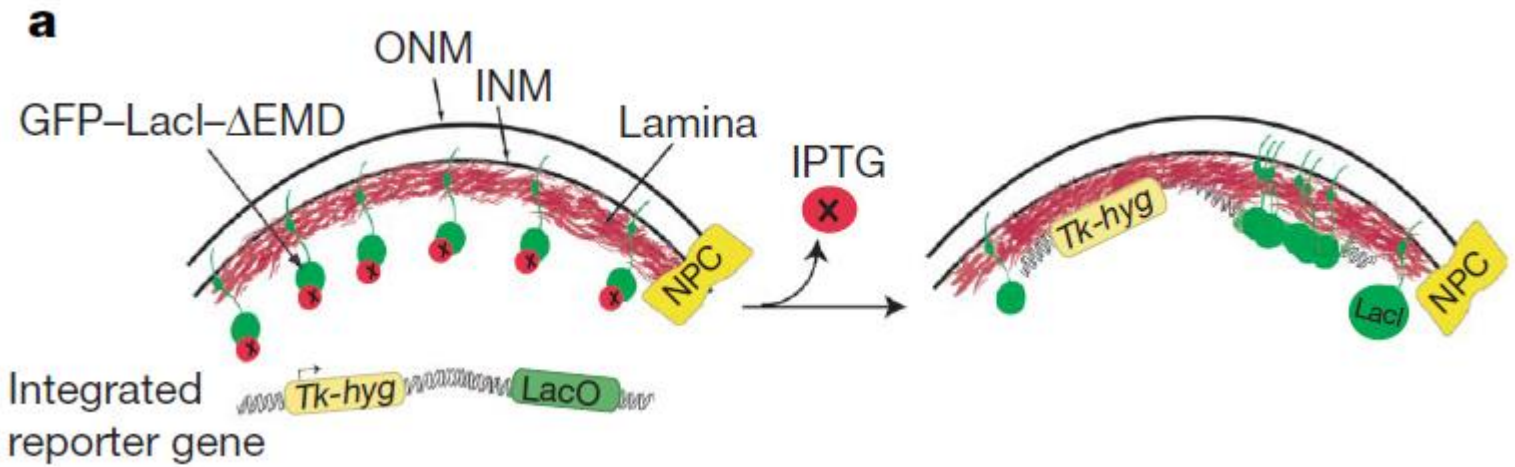
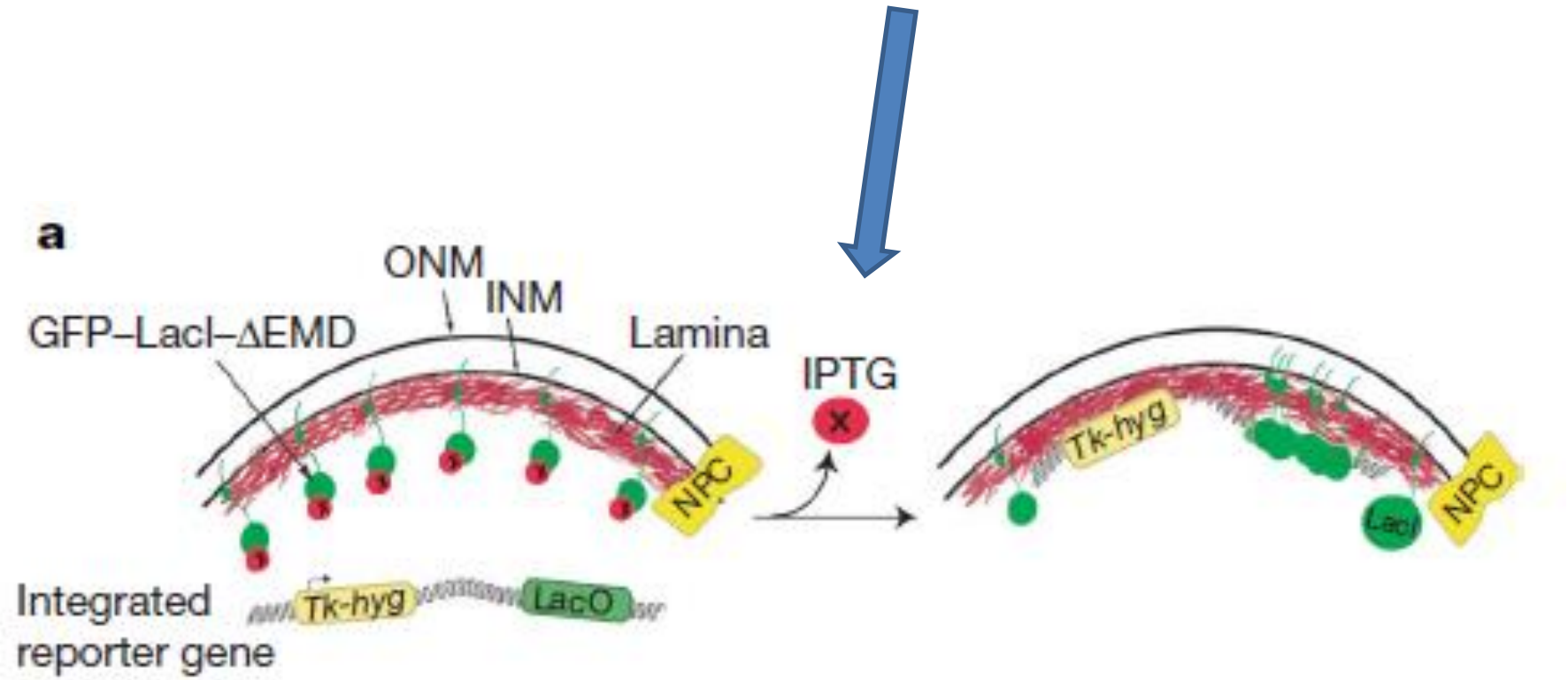


Figure Legend

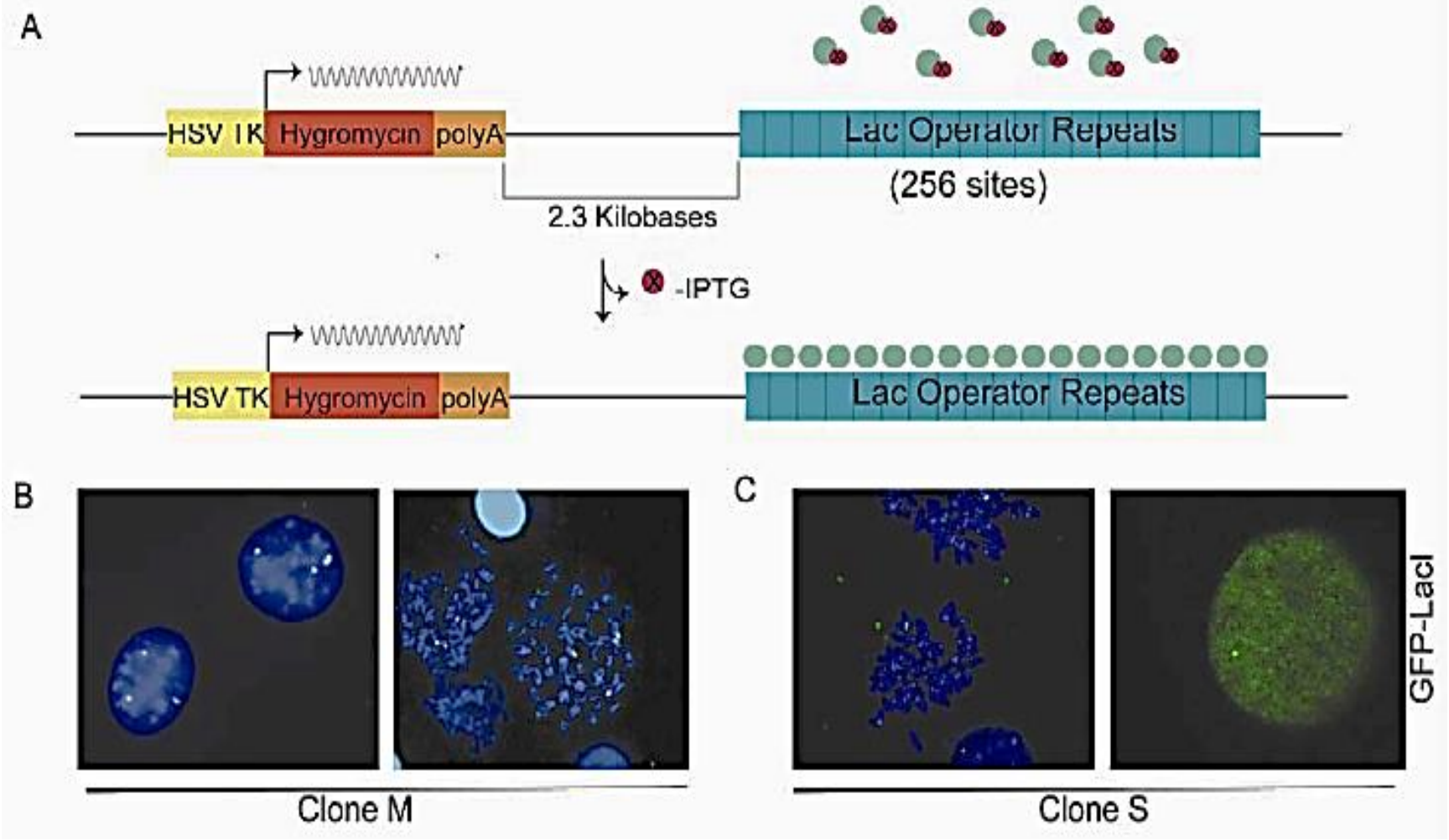
Figure 1 | Quantitative analysis of tethered loci by 3D DNA-immunoFISH. a, The stably integrated reporter gene contains an array of lac operators. The GFP–LacI–DEMD tethering protein is targeted to the INM and is unable to bind lacO sites in the presence of IPTG (red circles with cross in the centre). Upon withdrawal of IPTG, the tethering protein can bind to lacO sites in the integrated reporter gene construct. This interaction is anticipated to result in tight association of the reporter gene with the INM. (see also Supplementary Fig. 1e). ONM, outer nuclear membrane.

Upon IPTG removal, LacI will bind to its operator



What is this ?

E. Coli Lac Operon regulation

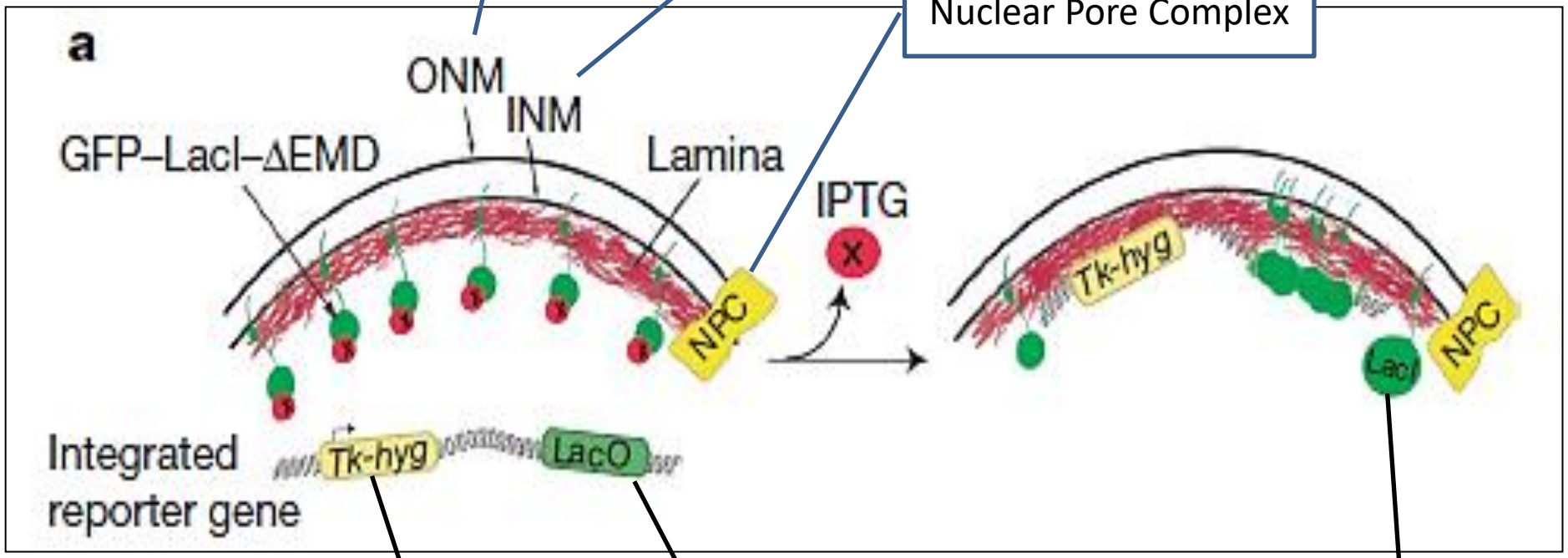

A
HSV TK
Hygromycin
polyA
Lac Operator Repeats
(256 sites)
2.3 Kilobases
-IPTG
HSV TK
Hygromycin
polyA
Lac Operator Repeats
B
Clone M
C
Clone S
GFP-LacI

ONM = Outer nuclear membrane

INM = Inner nuclear membrane proteins: LBR, LAP2, emerin

orienting labels

Nuclear Pore Complex

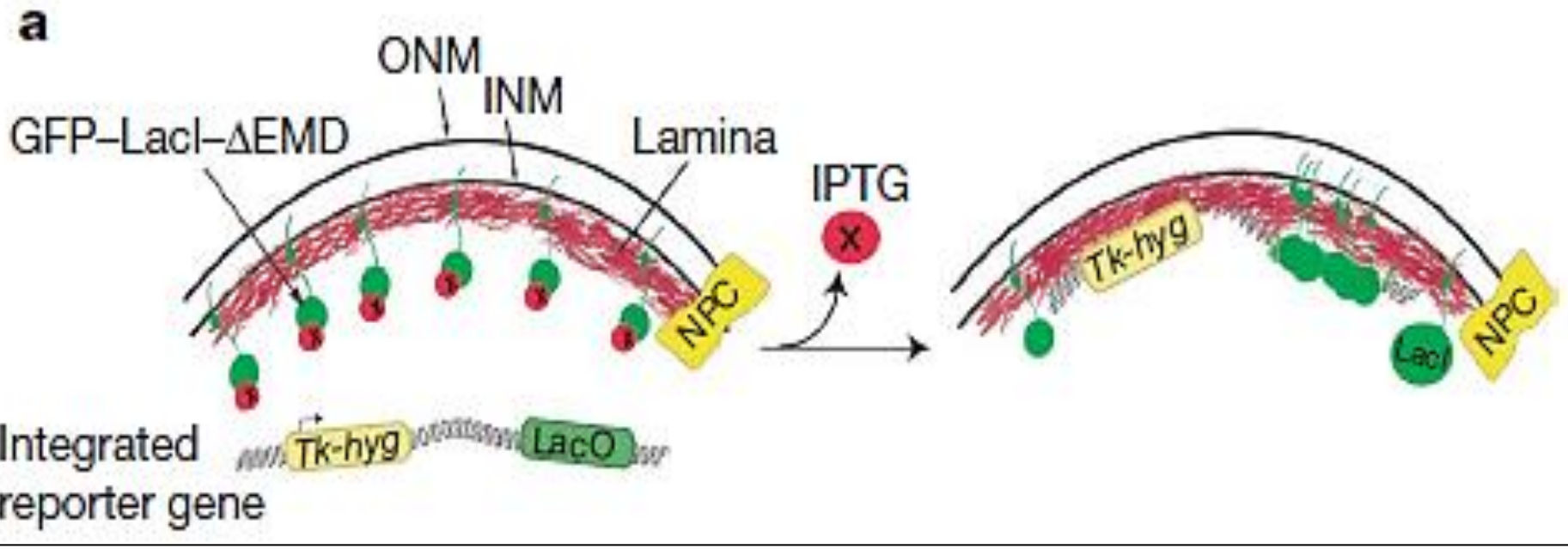


Herpes simplex virus Thymidine kinase promoter

E. Coli Lac operon Operator, multiple copies

Lac Repressor

System components



ΔEMD=emerin
C-term domain

GFP

LacI

2 fusion proteins

What is this used for ?

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.

Check initial clones

Check secondary clones with reporter

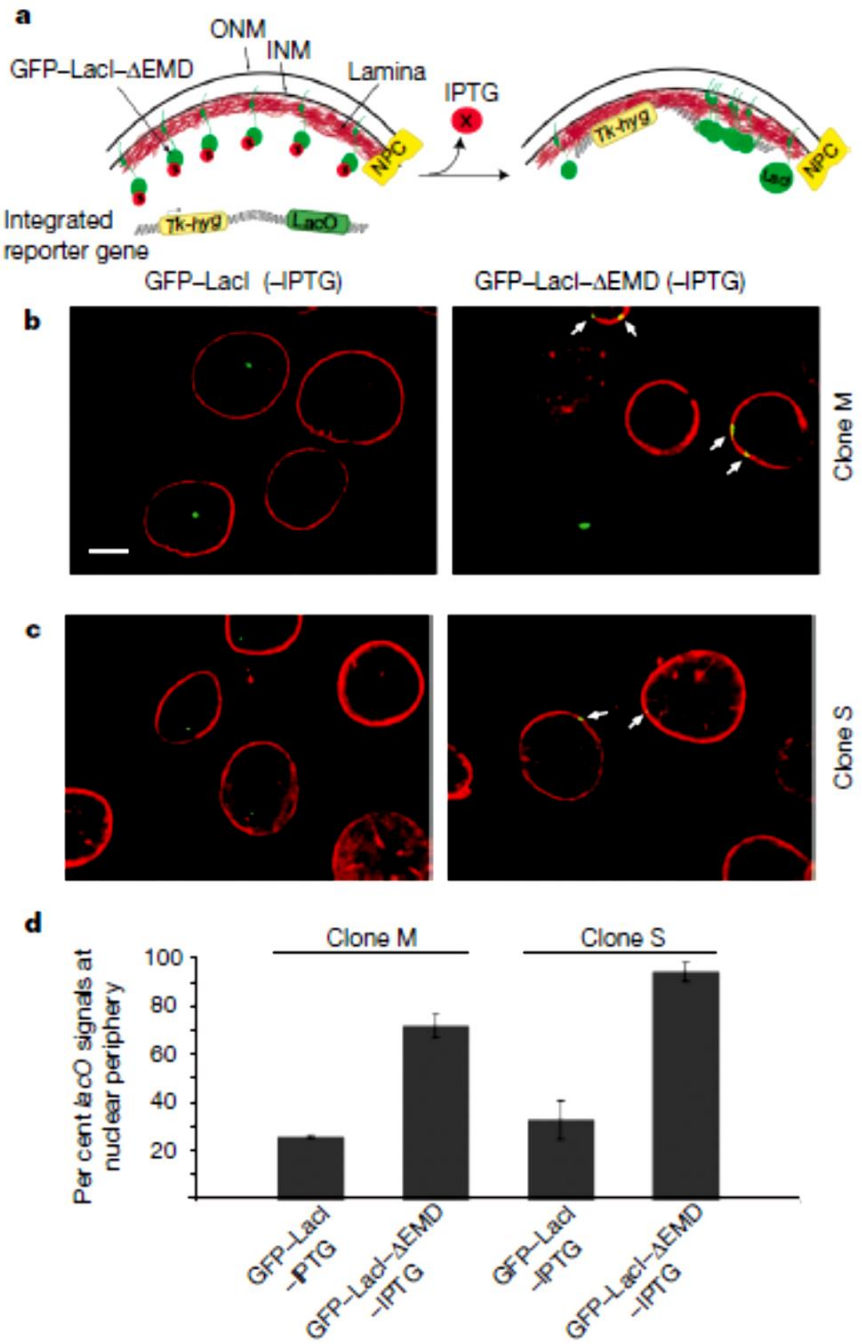
GFP results in Supplementary

GFP-LacI gives low signal

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

FISH

Control vector



INM-targeting vector

Figure 1.

Requires at least one cell division

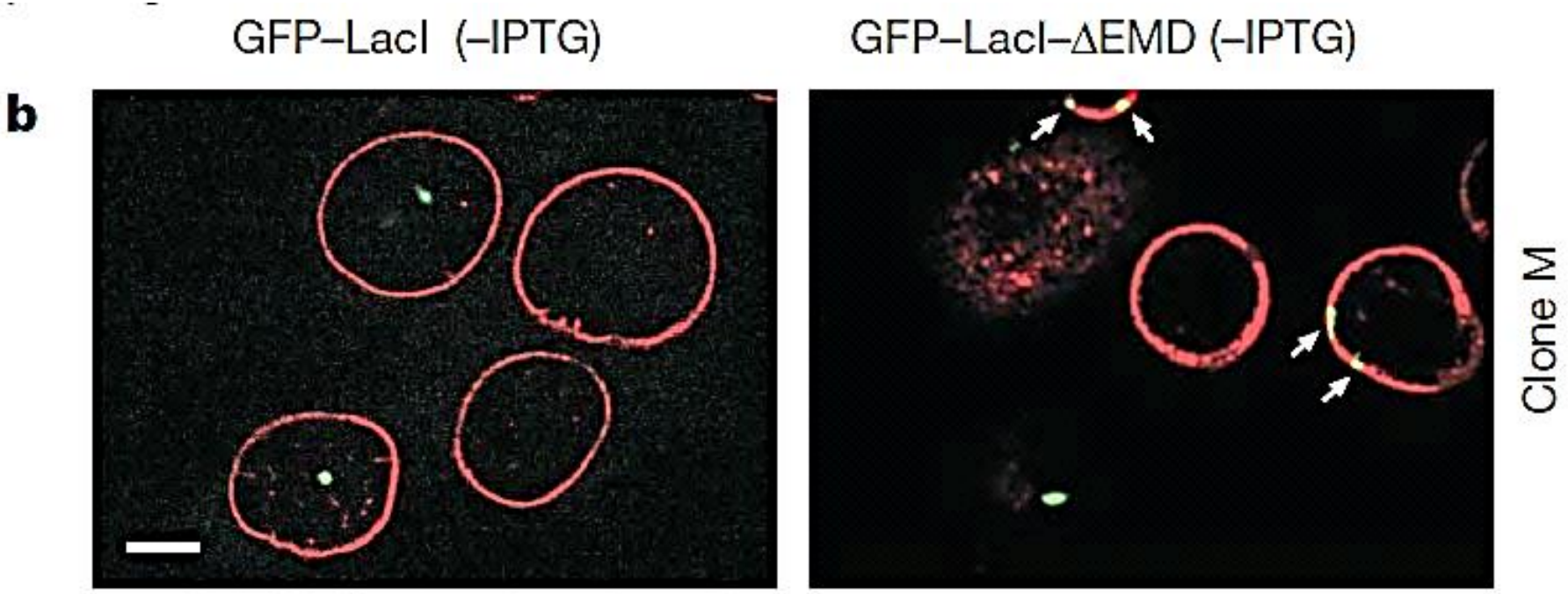


Figure 1 | Quantitative analysis of tethered loci by 3D DNA-immunoFISH.
b, Positioning of integrated reporter genes in clone-M nuclei detected by 3D DNA-immunoFISH. Shown is the nuclear distribution of lacO-bearing reporter genes in clone-M cells expressing GFP–LacI (left), and the repositioning of reporter genes in clone-M cells expressing GFP–LacI–DEMD upon IPTG withdrawal (24 h, right). Two confocal image planes of the same sets of nuclei are shown. In a and b, arrows mark co-localization of the lacO FISH signals with the nuclear lamina. Scale bar, 5 µm.

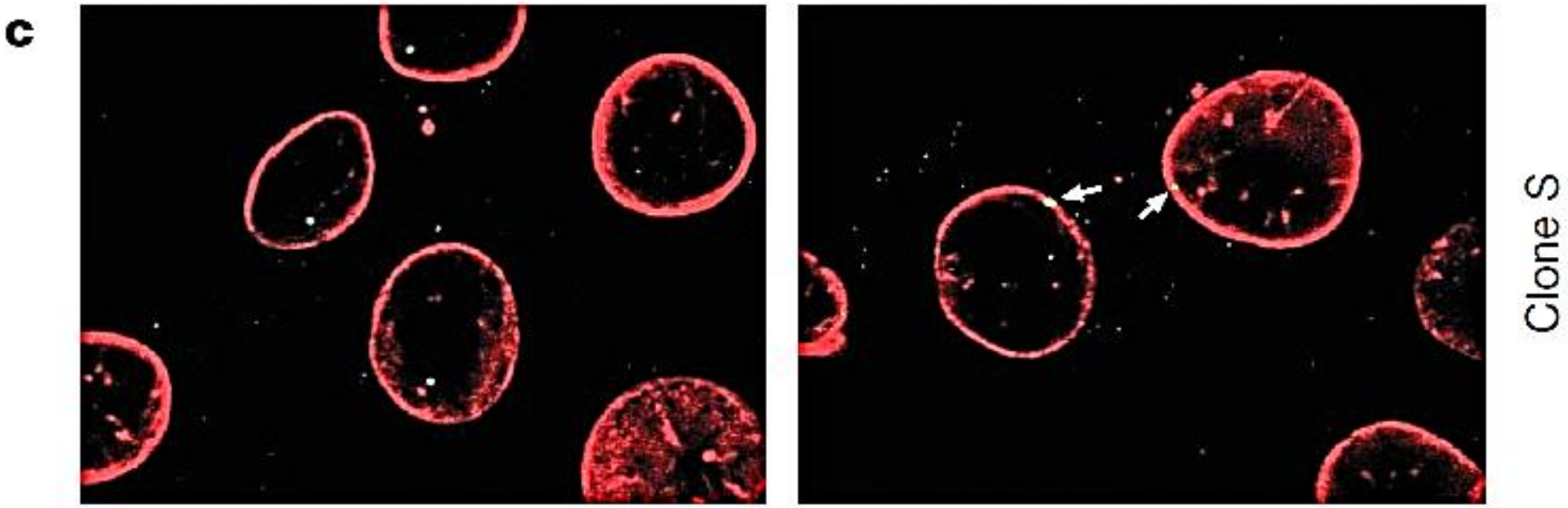
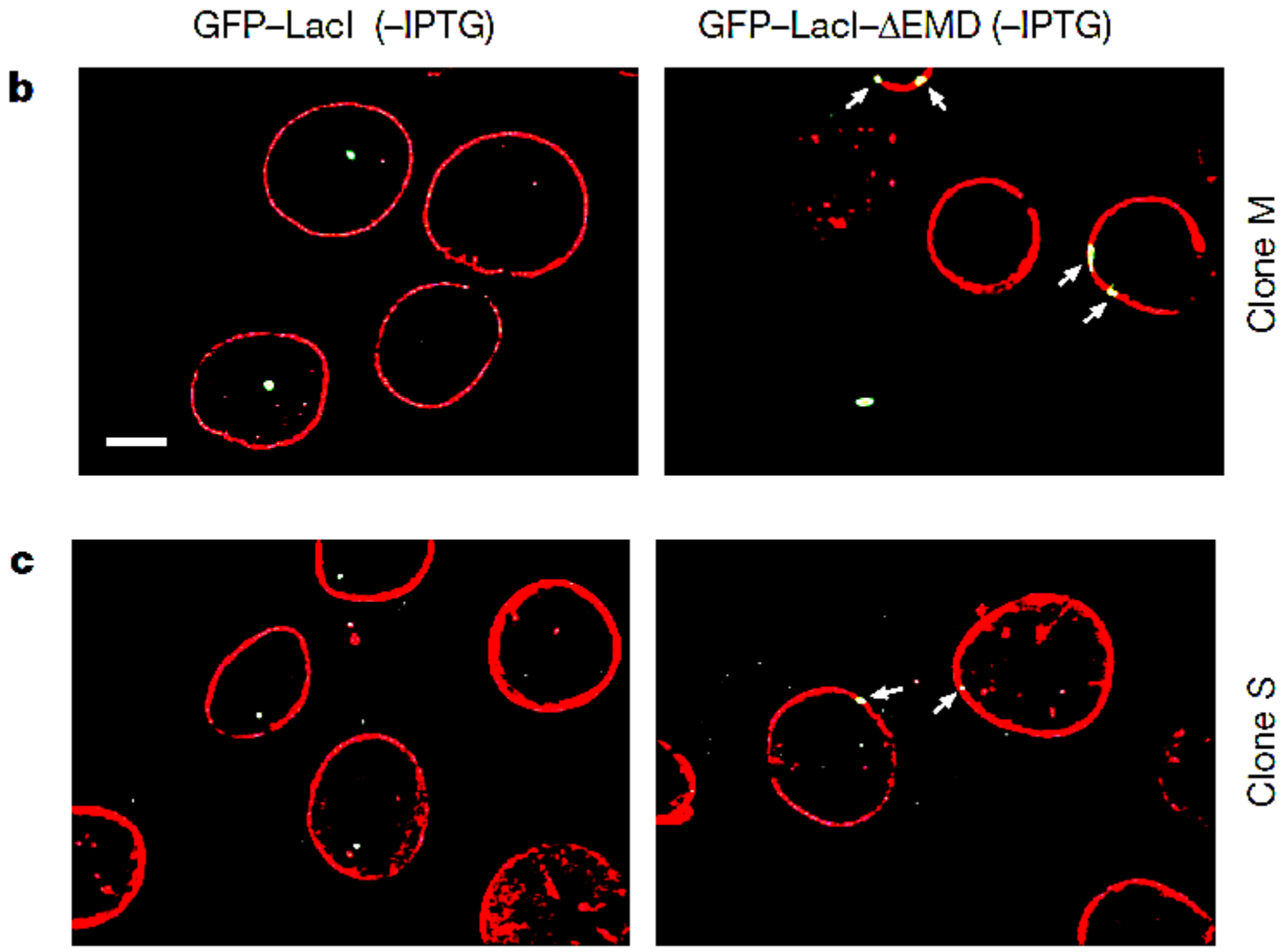
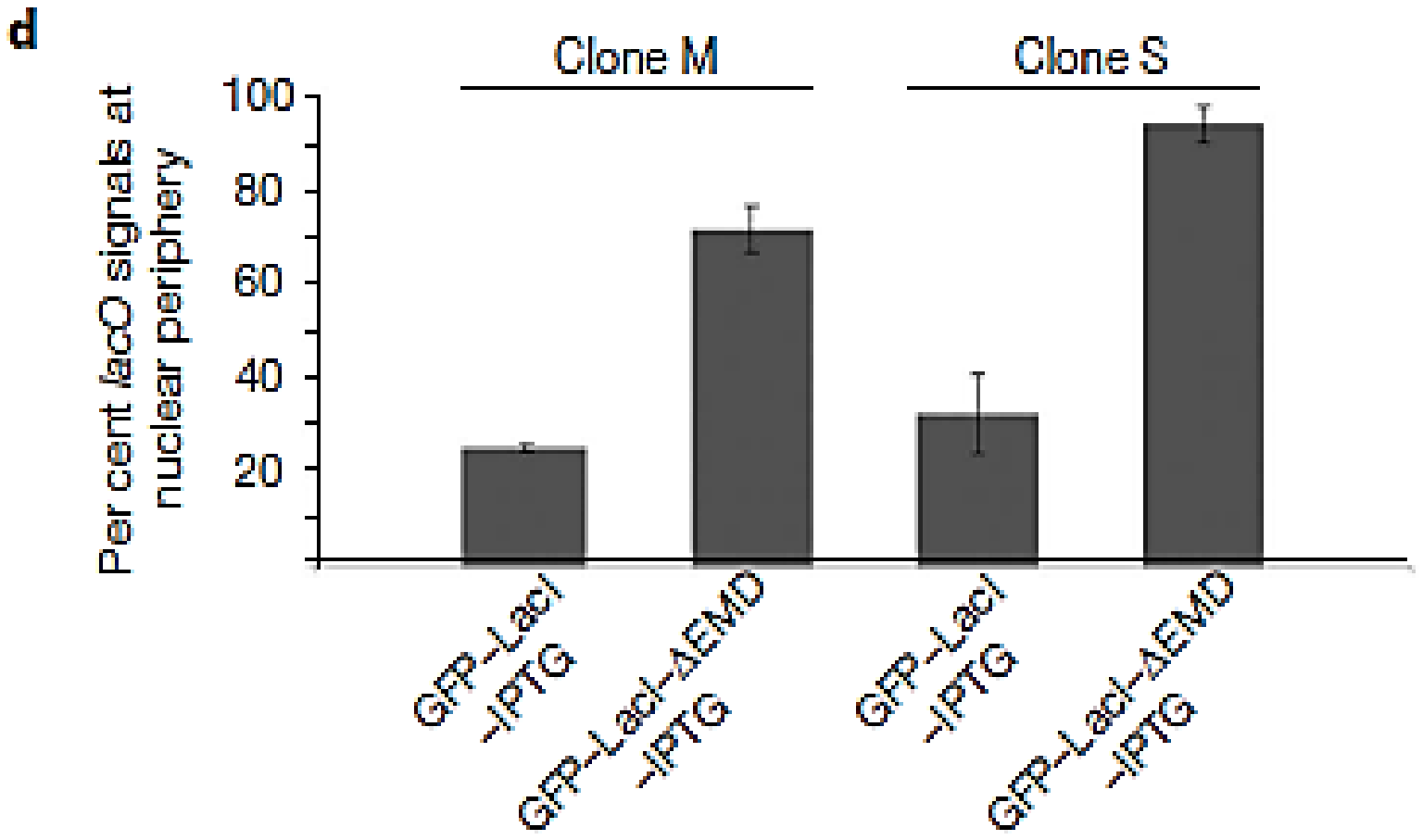


Figure 1 | Quantitative analysis of tethered loci by 3D DNA-immunoFISH.
c, Positioning of integrated reporter genes in clone-S nuclei. Single confocal planes of clone S cells expressing either GFP–LacI (–IPTG) or GFP–LacI–ΔEMD(–IPTG) are shown.


GFP–LacI (–IPTG)
GFP–LacI–ΔEMD (–IPTG)
b
Clone M
c
Clone S



d, The percentage of lacO signals at the nuclear periphery was determined by co-localization with LMNB1. In a given nuclear volume, lacO and LMNB1 signal intensities were converted to histograms. lacO signals were scored as peripheral if their peak intensity overlapped with LMNB1 (see Supplementary Fig. 2). The standard error bars indicate the deviation between two experiments under the indicated conditions. In a given experiment, either 50 (clone M) or 30 (clone S) nuclei were analysed for each condition.

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.

Conclusions for the first part

Part II – what is the consequence of tethering ?

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

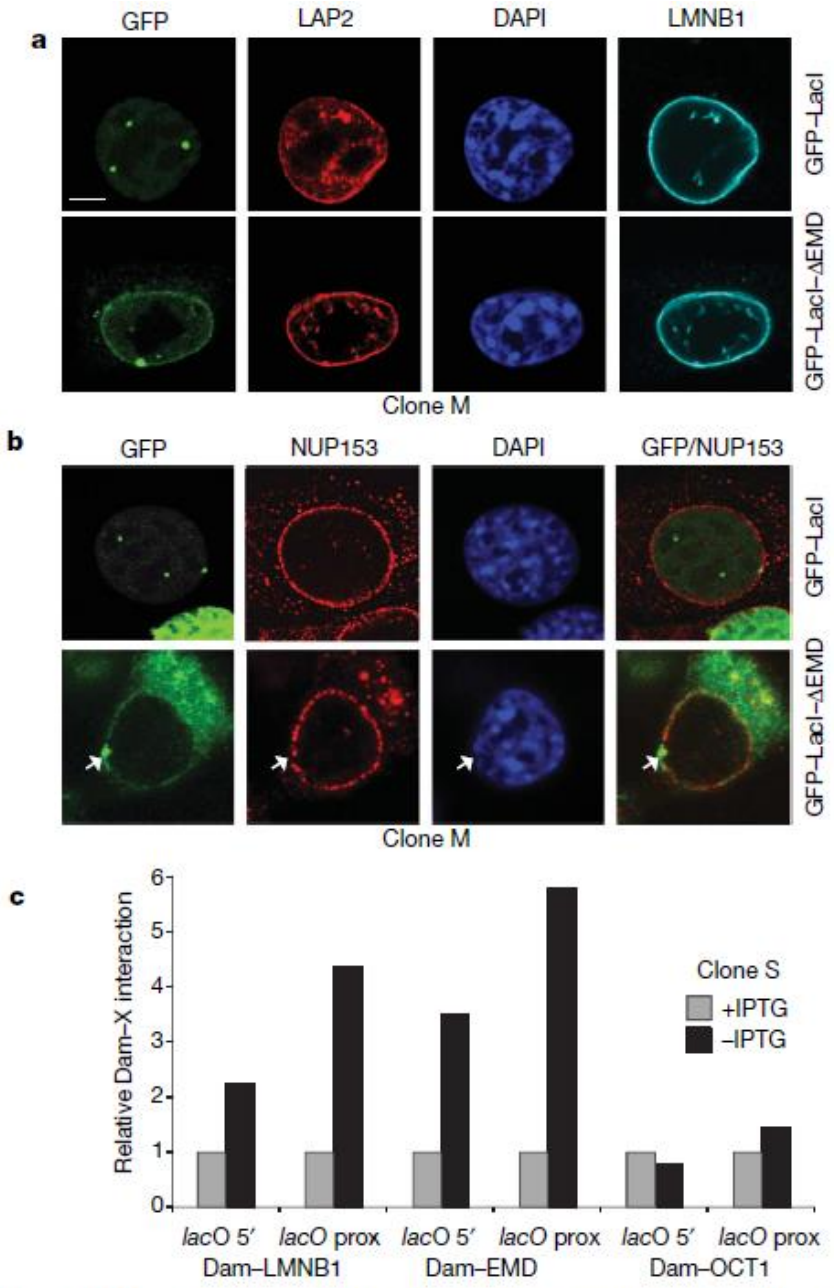


Figure 2 | Lamin B, EMD and LAP2, but not NPCs, accumulate at sites of tethered foci. a, b, Shown are *lacO* insertions detected by GFP fluorescence

CO-localization of transgene with Lamins and heterochromatic proteins

Dam-ID assay demonstrates direct interaction of Lamin B and Emerin with transgene

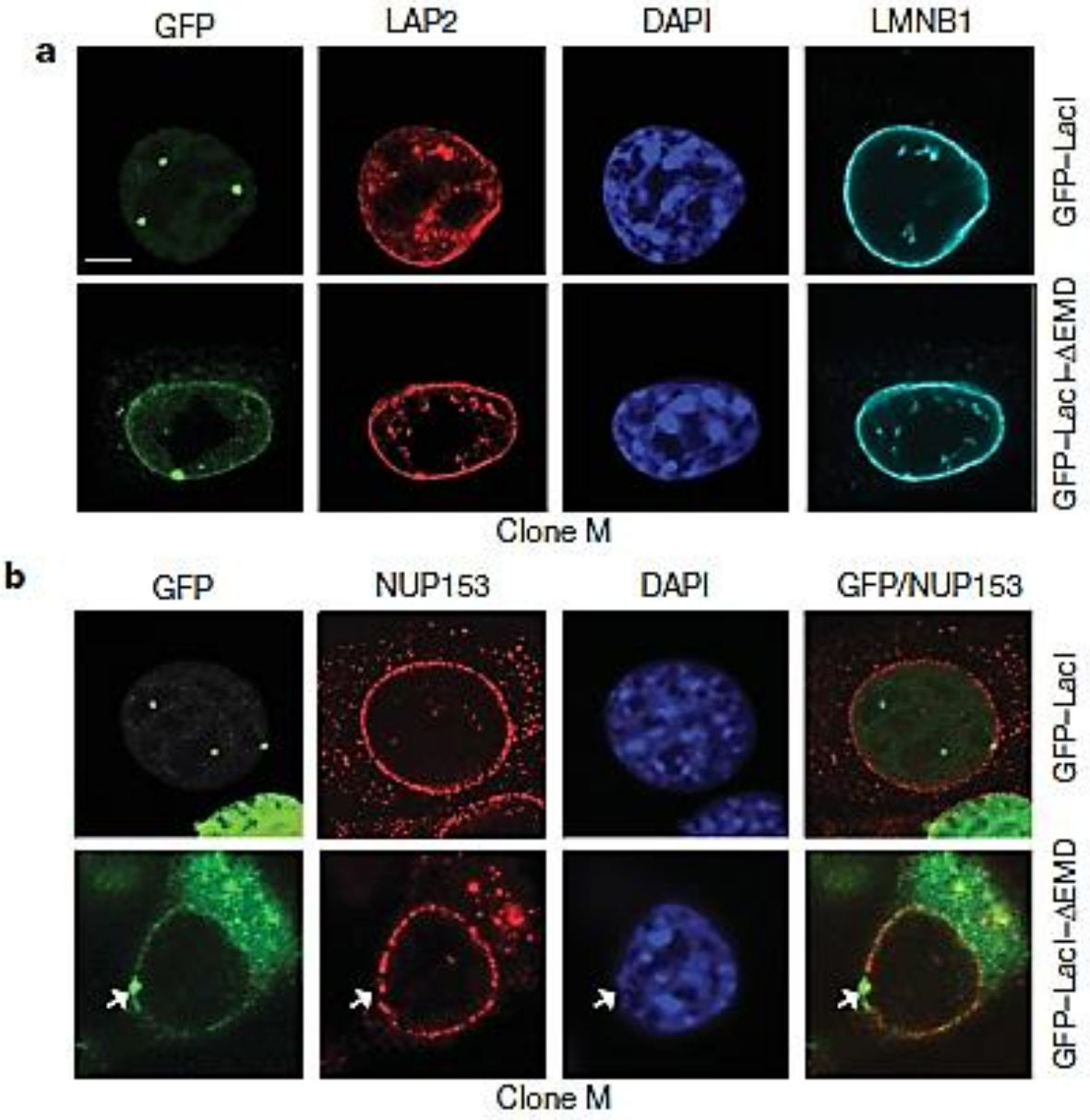


Figure 2 | Lamin B, EMD and LAP2, but not NPCs, accumulate at sites of tethered foci. a, b, Shown are lacO insertions detected by GFP fluorescence in clone-M cells expressing either GFP–LacI or GFP–LacI–DEMD upon IPTG withdrawal (24 h). Antibodies directed against LMNB1 (a, cyan), LAP2 (a, red) or NUP153 (b, red) were used to analyse accumulation of these components at sites of tethered lacO foci (arrows). The DNA-specific dye, DAPI (4',6-diamidino-2-phenylindole), was used to stain the nuclei. All images shown are single confocal sections. Arrows mark tethered loci. Scale bar, 5 µm.

LaminB1 and Lamin-Associated Protein2 accumulated at sites of tethering

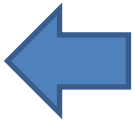
NUP153=component of the nuclear pore complex

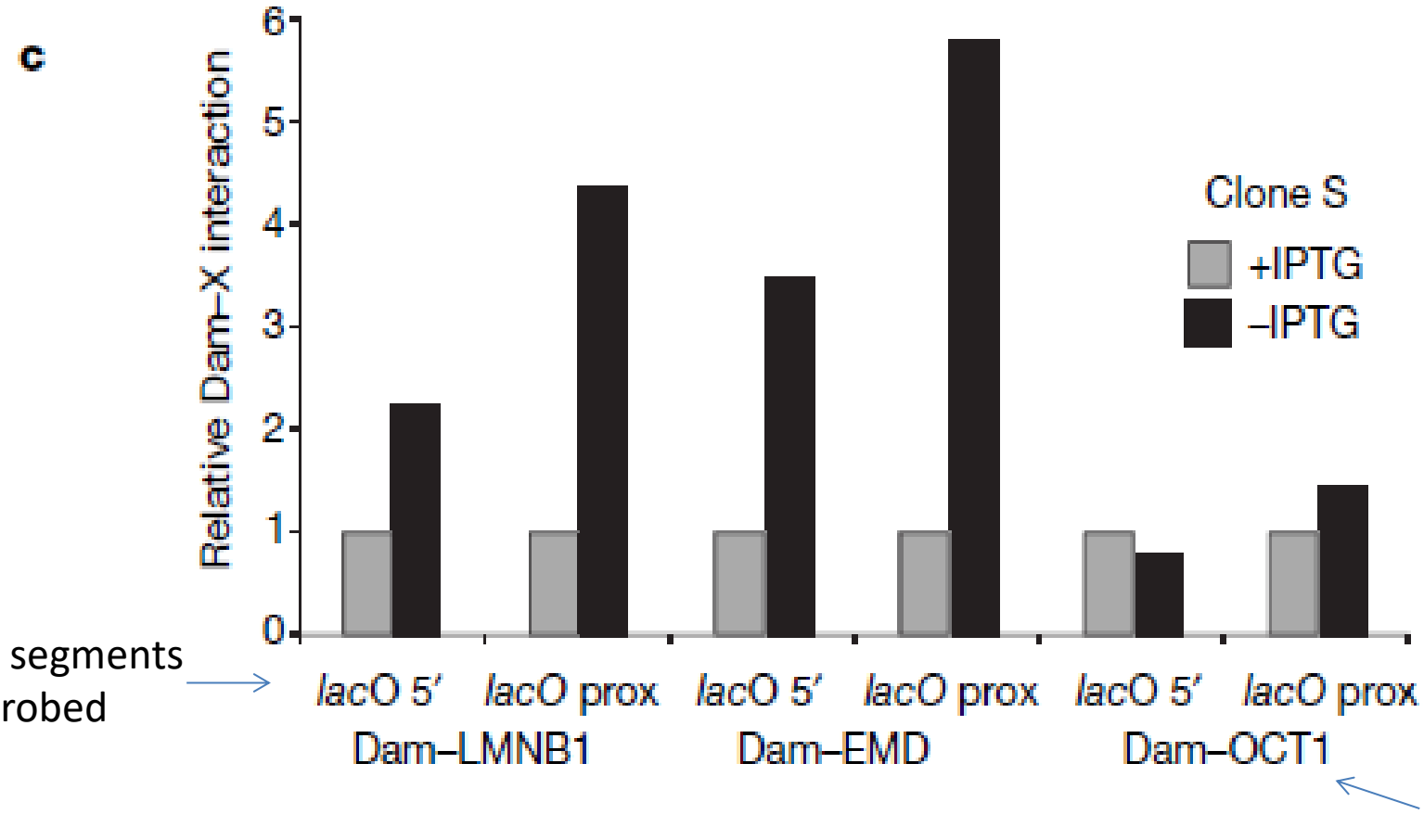
Co-localizaton is just «regional» (due to limited resolution of fluorescence microscopy) or does it involve true molecular proximity ?

The question needs a «proximity» assay.
Dam-ID is perfect to this purpose (see Lecture 2).

DAM-ID assay for proximity

The accumulation of INM–lamina components at sites of tethering may not necessarily reflect molecular interactions with the underlying chromosomal DNA. Furthermore, in clone-S cells, the accumulation of INM and lamin proteins could not be monitored by means of immunofluorescence. Therefore, we used the DamID methodology to detect INM–lamina protein interactions with the test gene upon tethering[18]. EMD and lamin B1 (LMNB1) fusion proteins containing the *E. coli* DNA adenine methyltransferase (Dam) were expressed in clone-M and close-S cells. We detected an increase in Dam–EMD- and Dam–LMNB1-mediated methylation of the reporter genes upon tethering (Fig. 2c and Supplementary Fig. 4b). Collectively, these data show that tethering of a chromosomal segment to the INM facilitates the localized recruitment of lamin and INM proteins to the DNA.





2 segments probed

Go to UniProt

?

c, Molecular interactions of LMNB1, EMD and OCT1 with the reporter gene, detected by DamID-mediated methylation, in clone-S nuclei. All interactions were normalized to the signal from cells transduced with Dam alone. Untethered test genes (+IPTG) were set to one after normalization; the y-axis indicates the fold change in the Dam–X signal (where X is LMNB1, EMD or OCT1) upon tethering (2IPTG). A representative experiment is shown. lacO 59 and lacO prox are PCR primer pairs that are positioned upstream or downstream of the lacO arrays, respectively.

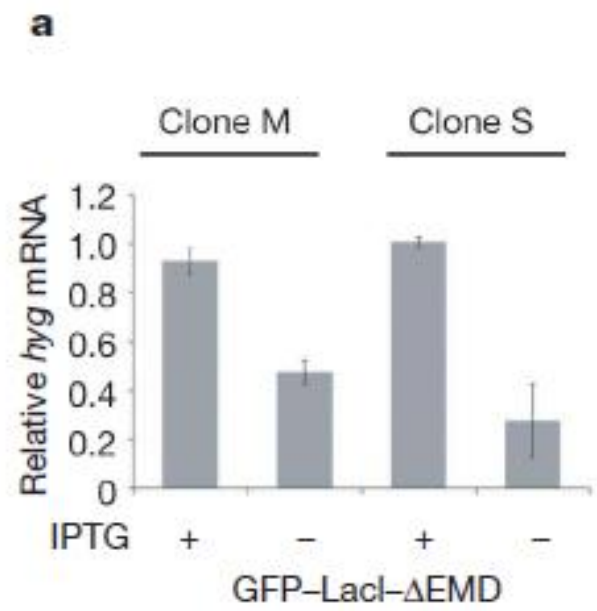
Part 3 – what is the consequence on gene activity ?

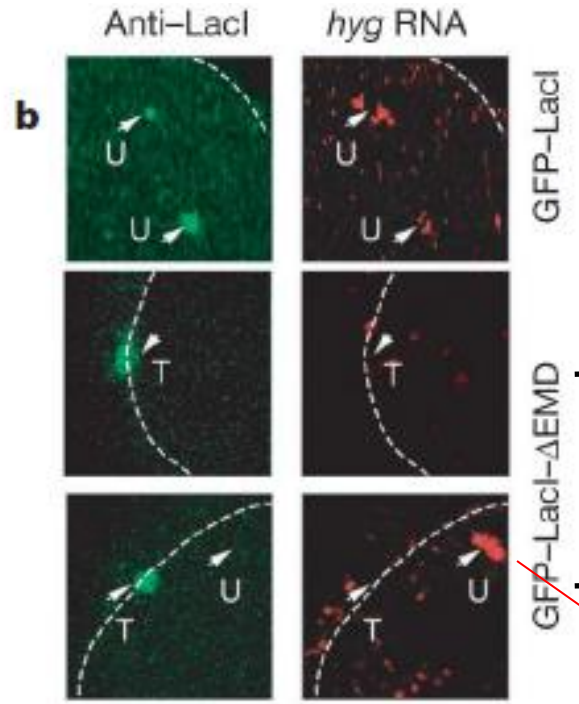
qRT-PCR

To test the consequence of repositioning of reporter genes to the INM, we analysed transcript levels of *hyg* (Fig. 3a and Supplementary Fig. 6). Upon tethering, *hyg* gene activity was repressed in clone-M and clone-S cells. Importantly, binding of nucleoplasmic GFP–LacI molecules to *lacO* sites in the reporter construct did not impair *hyg* expression (Supplementary Fig. 6). The EMD segment (Δ1–64) used in tethering lacks a domain required for interaction with transcriptional repressors[19]. Nevertheless, to rule out the possibility that this segment was mediating repression in the absence of repositioning to the nuclear lamina, we generated a nucleoplasmic version that lacks the C-terminal transmembrane domain necessary for targeting to the INM (GFP–LacI–ΔEMD*). Importantly, GFP–LacI–ΔEMD* did not repress the *hyg* gene

control

May Emerin function as a repressor ?



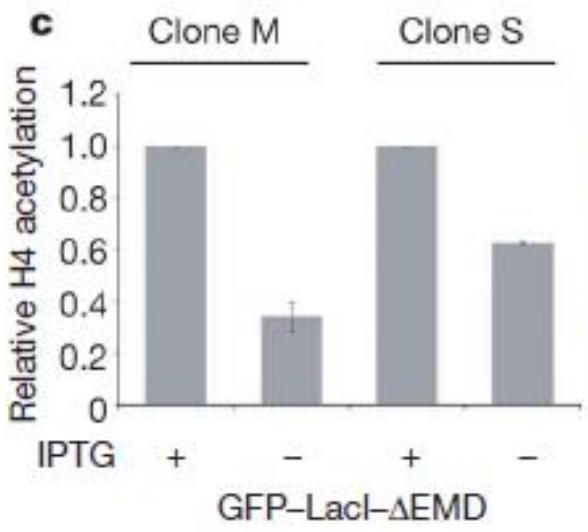


control

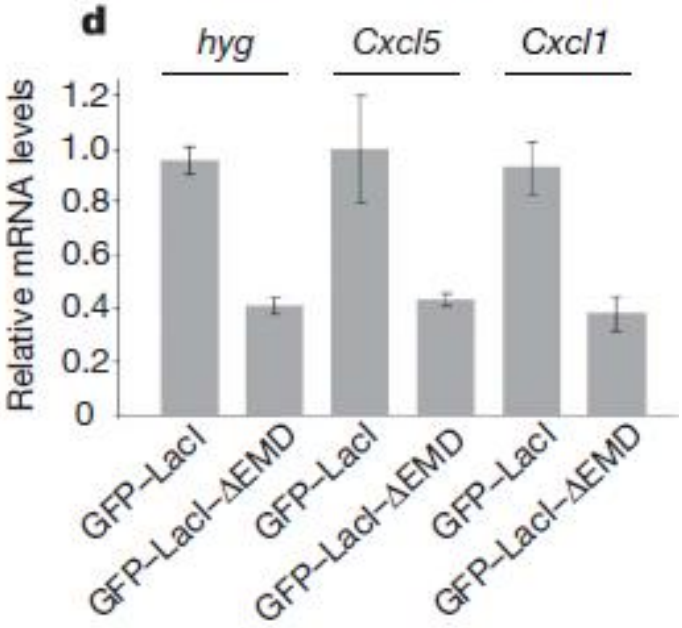
tethered

untethered

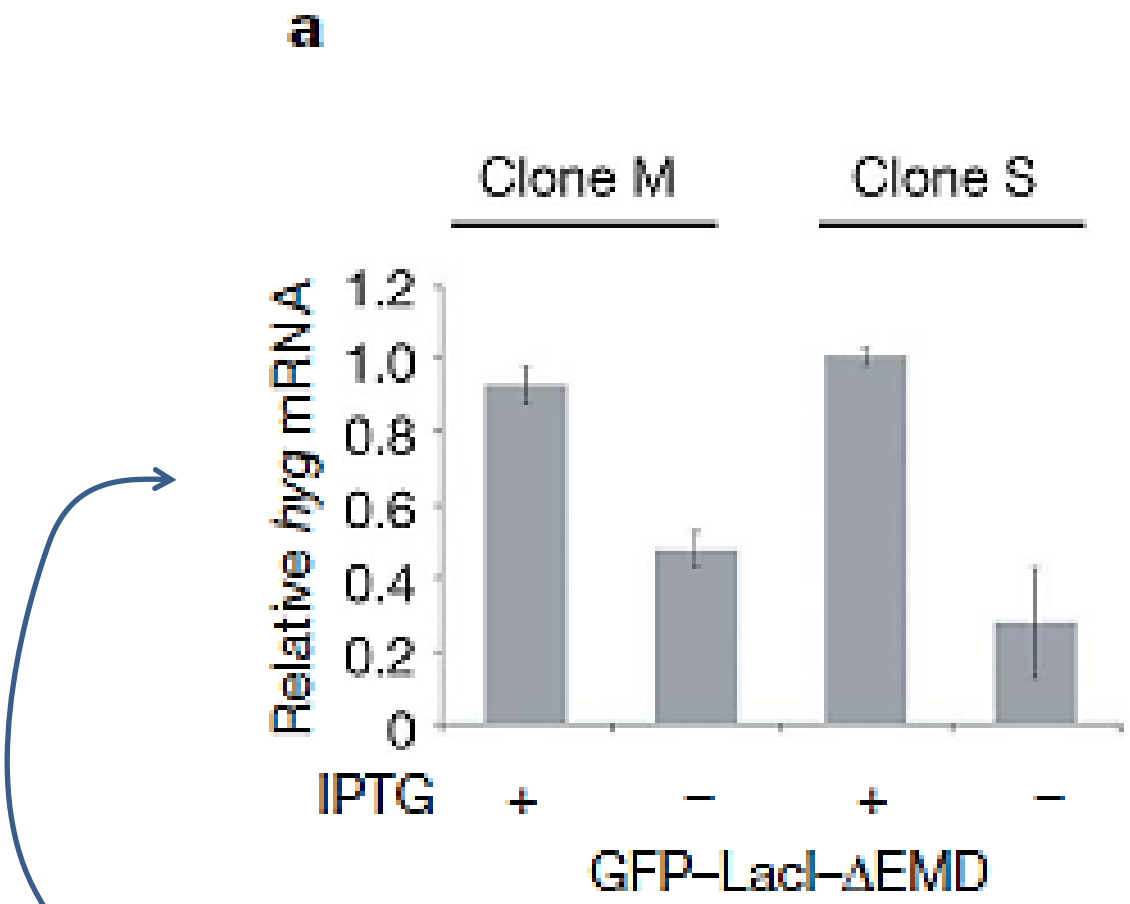
Nascent hyg RNA is red



Histone acetylation



Neighbours expression by Q-RT-PCR

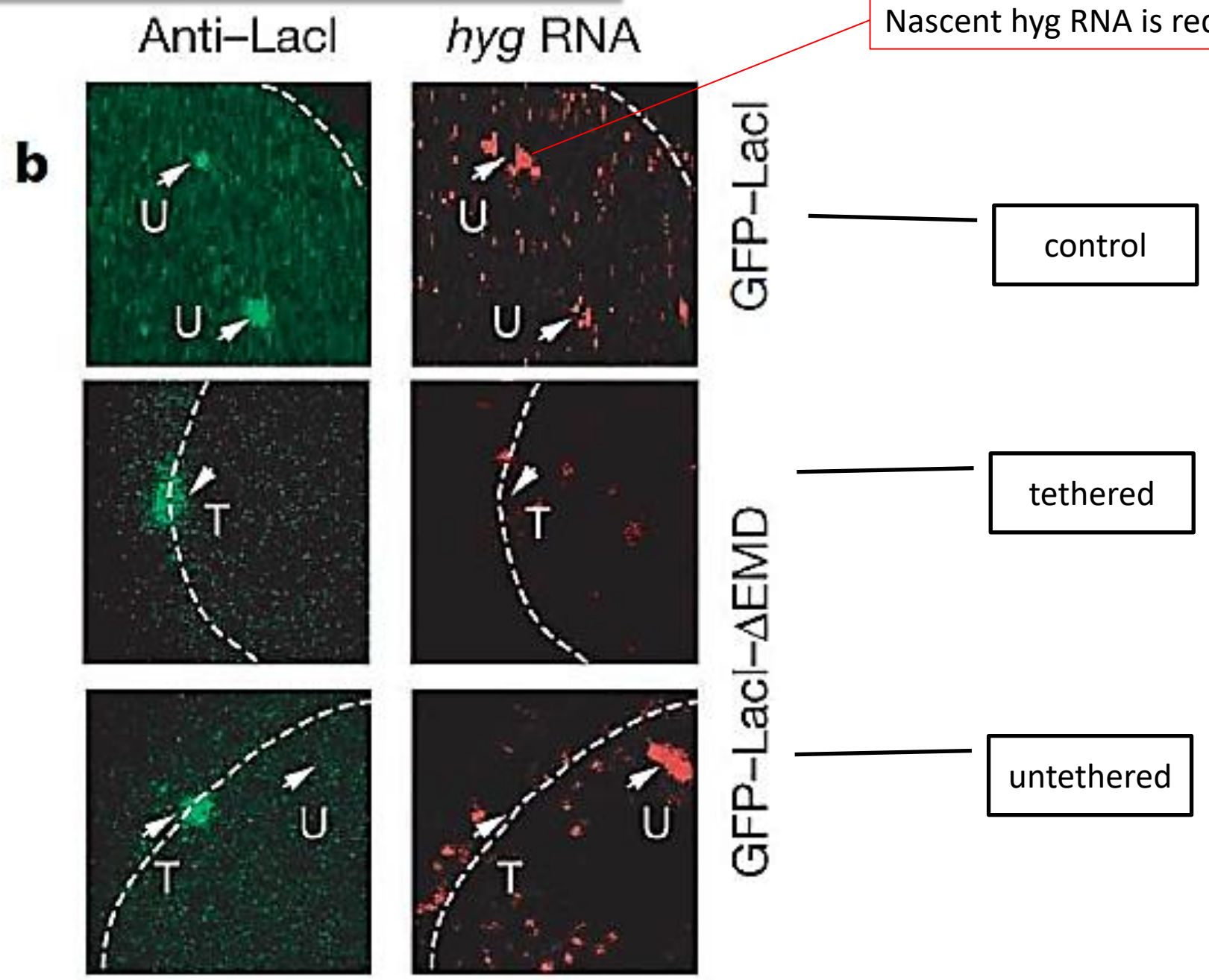


Hyg mRNA measured by extracting total RNA from cells, then performing a «retrotranscription» (→cDNA) using Reverse Transcriptase. The cDNA was then analysed using real-time fluorescent quantitative PCR.
The method is: quantitative RetroTranscription PCR (qRT-PCR).
The axis label says «Relative» since expression is normalized to one sample (in this case, the highest one).

Figure 3b

not repress the *hyg* gene (Supplementary Fig. 6). We next performed single-cell analysis using 3D RNA-immunoFISH. This enabled direct comparison of the transcriptional activity of untethered versus tethered loci. Most of the GFP–LacI foci (70%) were associated with *hyg* RNA signals (Fig. 3b). In contrast, most of the tethered, GFP–LacI–ΔEMD-bound loci (80%) showed no or reduced *hyg* RNA signals. We note that in GFP–LacI–ΔEMD-expressing cells, not all loci are tethered (Fig. 1d), and consequently *hyg* signals were observed emanating from them (Fig. 3b). Collectively, these results demonstrate that the test gene undergoes transcriptional repression as a consequence of repositioning to the INM.

Three-dimensional RNA-immmunoFISH



Nascent hyg RNA is red

control

tethered

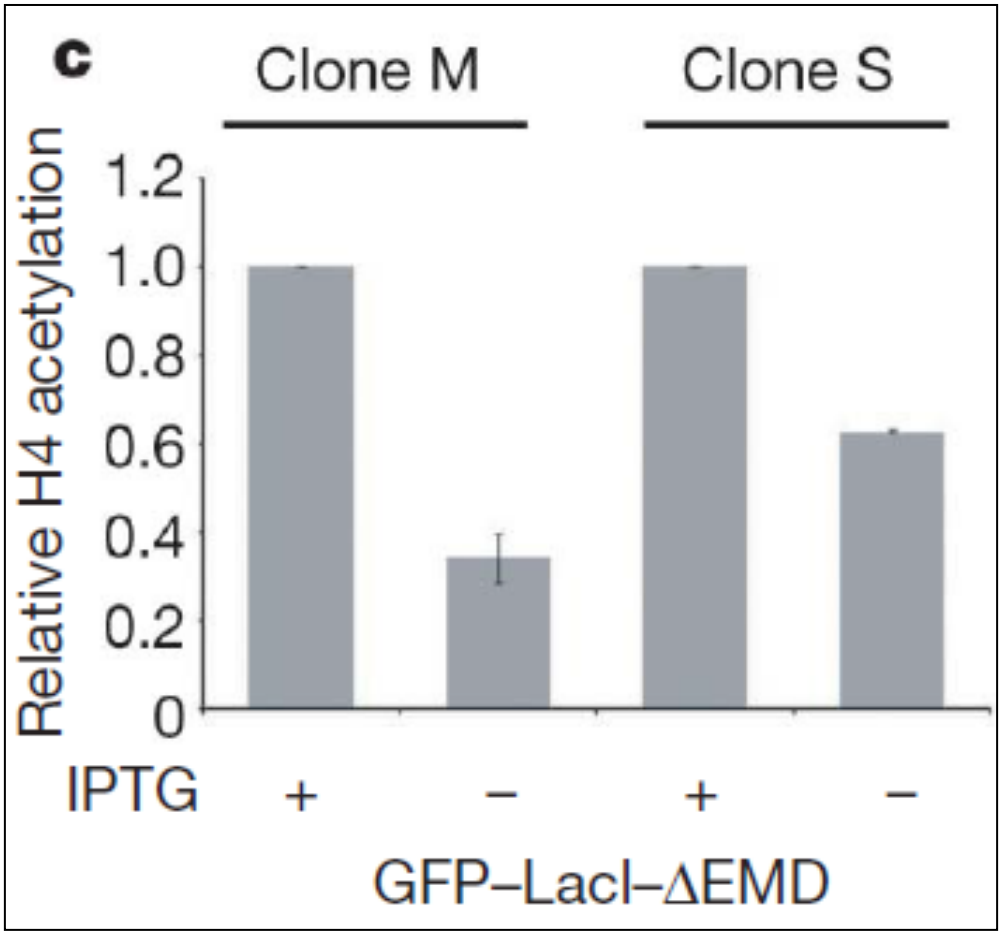
untethered

Given the evidence that genes associated with the nuclear lamina are hypo-acetylated and that LAP2β interacts with HDAC3, we determined the consequences of tethering on the H4 acetylation status of our reporter gene (Fig. 3c)[12,20]. The untethered promoter region displayed a high degree of acetylation. Upon tethering, a decrease in histone H4 acetylation was observed. Thus, the transcriptional repression caused by tethering of a gene to the INM is accompanied by histone H4 hypo-acetylation.

Histone acetylation

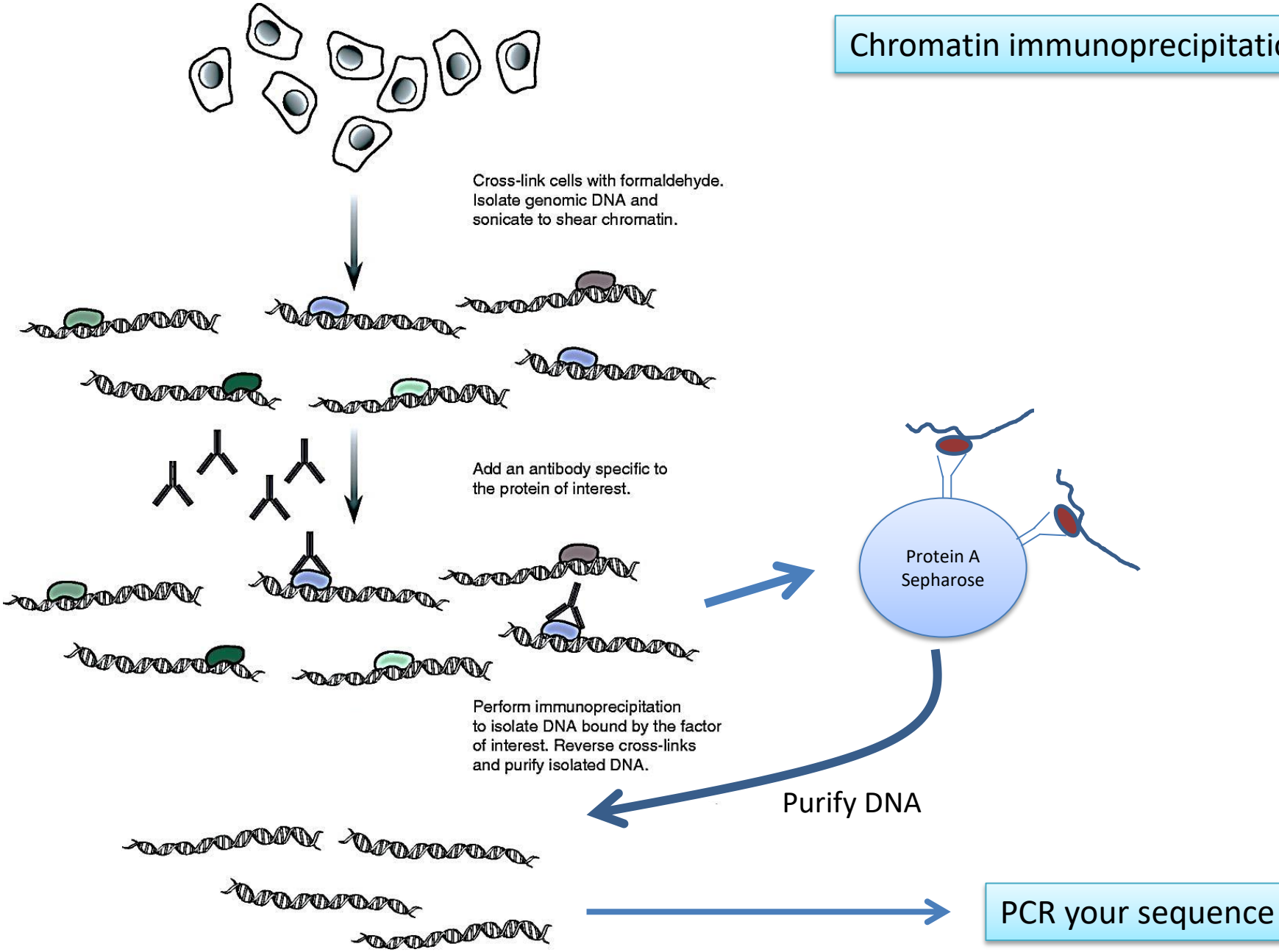
By ChIP analysis

Chromatin immunoprecipitation followed by quantitative PCR



Primers on the HSVTK - promoter region

Chromatin immunoprecipitation



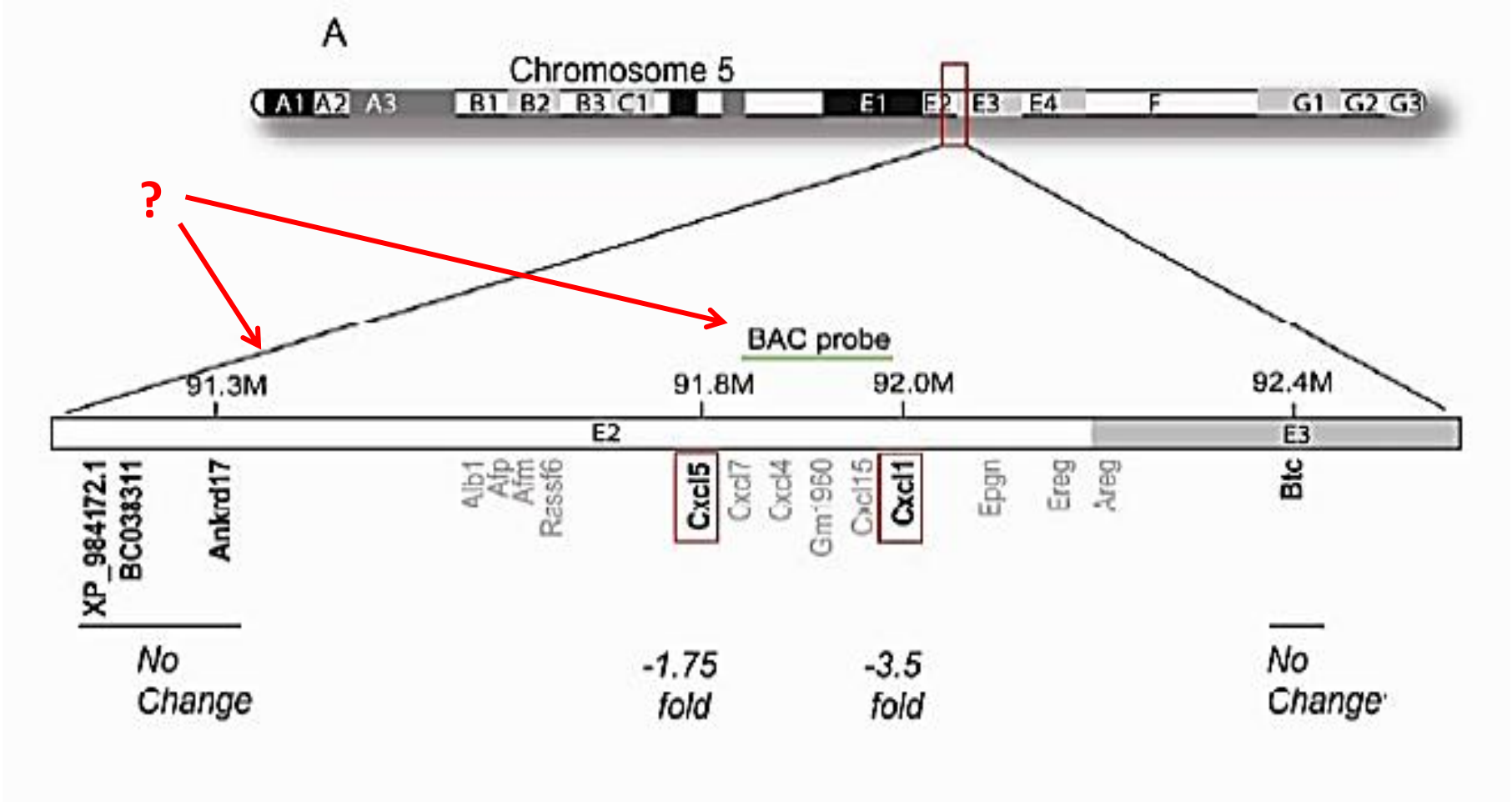
PCR your sequence

We next explored if endogenous genes flanking a *lacO* insertion site might also be repressed on relocalization to the INM; we did this by surveying clone-M cells using genome-wide expression analysis. We identified 51 genes that were repressed under tethering conditions including a pair on chromosome 5 (*Cxcl1* and *Cxcl5*, Supplementary Fig. 7a). Transcriptional repression of the *Cxcl1* and *Cxcl5* genes was verified by quantitative PCR (Q-PCR, Fig. 3d). Importantly, a bacterial artificial chromosome (BAC) probe covering this region co-localized with a *lacO* integration site (Supplementary Fig. 7b). In clone-S cells, the test gene is inserted 227 kb away from the nearest gene whose activity was unaffected by tethering (data not shown). These results show that endogenous flanking genes can be repressed by tethering to the INM, and suggest that a delimited inactive chromosomal domain may be generated around a site of attachment.

Conclusions to part 3

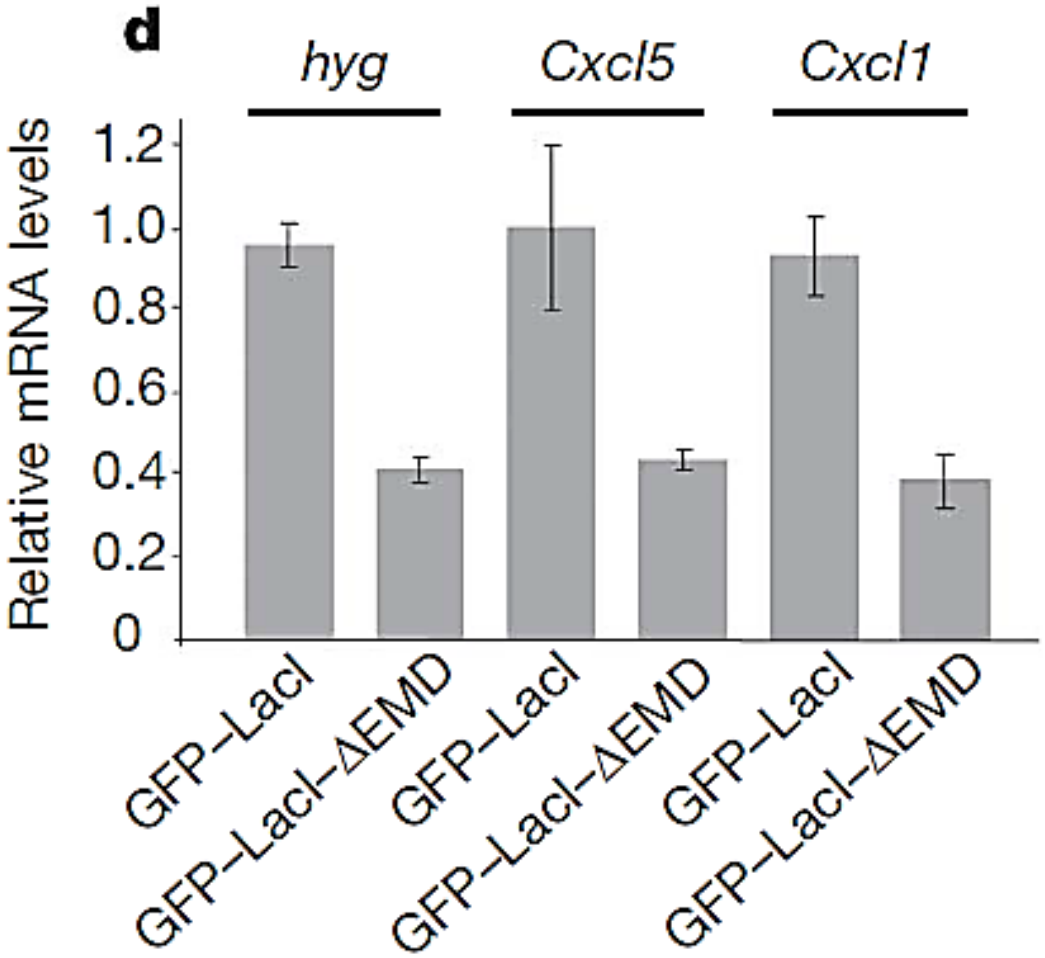
What is a BAC ?

Supplementary Figure S7 – mapping of the integration site in clone S



qRT-PCR analysis

Neighbouring genes



B

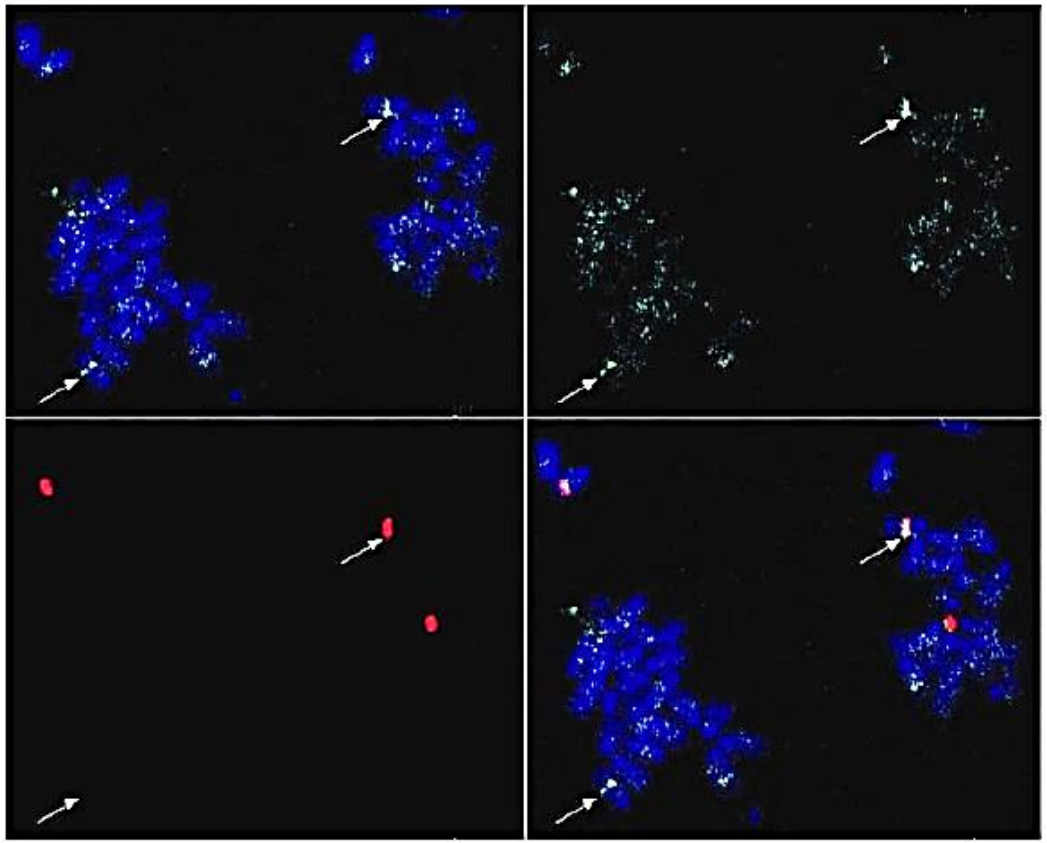


Fig. S7 One of the *lacO* insertions co-localize with the *Cxcl1* and *Cxcl5* genomic region in clone M cells (A) Schematic depicts a region of chromosome 5 that harbors a pair of chemokine genes (*Cxcl1* and *Cxcl5*) that are repressed under tethering conditions by microarray analysis. Genes in light grey are not expressed at detectable levels under any conditions (tethered or untethered). Genes in bold were expressed in the untethered cells and fold change is indicated under those genes. (B) 2D FISH analysis (BAC clone depicted in A) and a site of *lacO* insertion. The hybridization signal of the BAC clone is shown in green and the signal from the *lacO* insertions is shown in red.

Part 4 – Is this limited to transgenes or can be seen on endogenous genes as well ?

As is the case for our tethered test gene, transcriptionally inactive and hypo-acetylated immunoglobulin heavy chain (*Igh*) loci in NIH3T3 fibroblasts and in T cells are positioned at the nuclear lamina (Fig. 4a and data not shown)[3]. Therefore, we used the aforementioned Dam-fusion proteins to test if the *Igh* loci in NIH3T3 cells were in molecular contact with the INM–lamina. We note that the Dam–OCT1 (also known as POU2F1) fusion protein monitors the accessibility of immunoglobulin loci at the nuclear periphery because OCT1 is a transcription factor that binds to *VH* gene promoters and regulates their activity[21]. For DamID, we used primers spanning a domain of the *Igh* locus containing the *VHJ558* gene family, implicated in mediating association with the nuclear periphery[17,21]. Both LMNB1 and EMD were seen to interact with peripherally positioned *VH* genes in NIH3T3 nuclei (Fig. 4a). These interactions were not

There was a know case in the literature:

As is the case for our tethered test gene, transcriptionally inactive and hypo-acetylated immunoglobulin heavy chain (Igh) loci in NIH3T3 fibroblasts and in T cells are positioned at the nuclear lamina (Fig. 4a

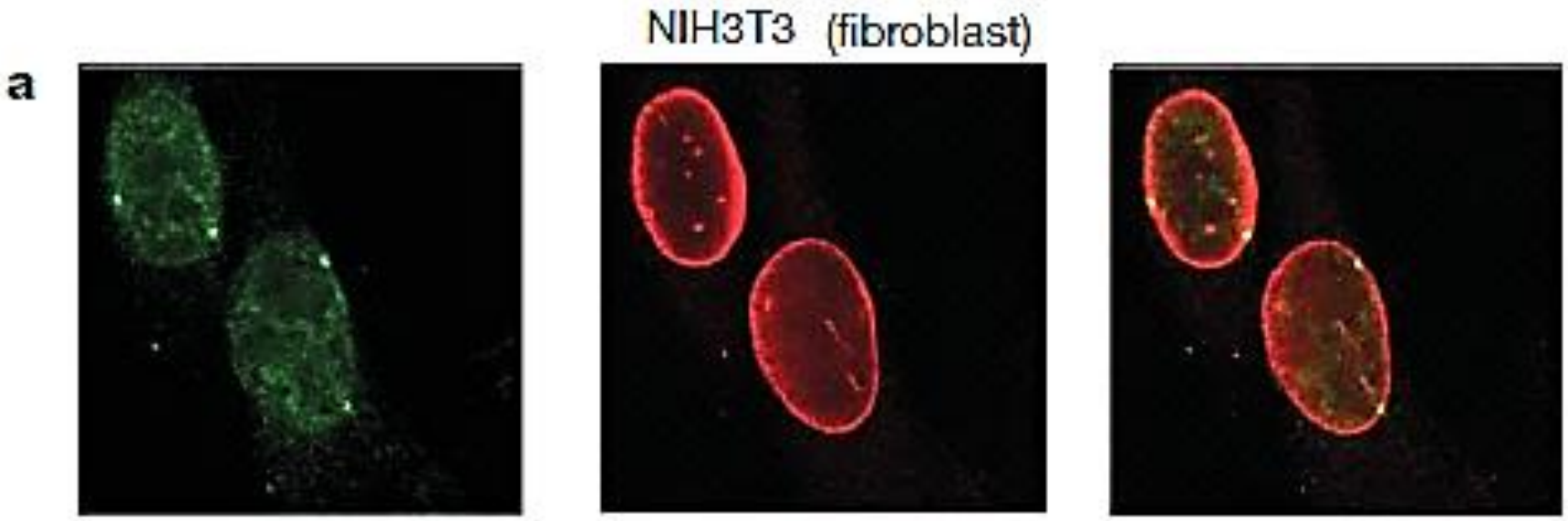
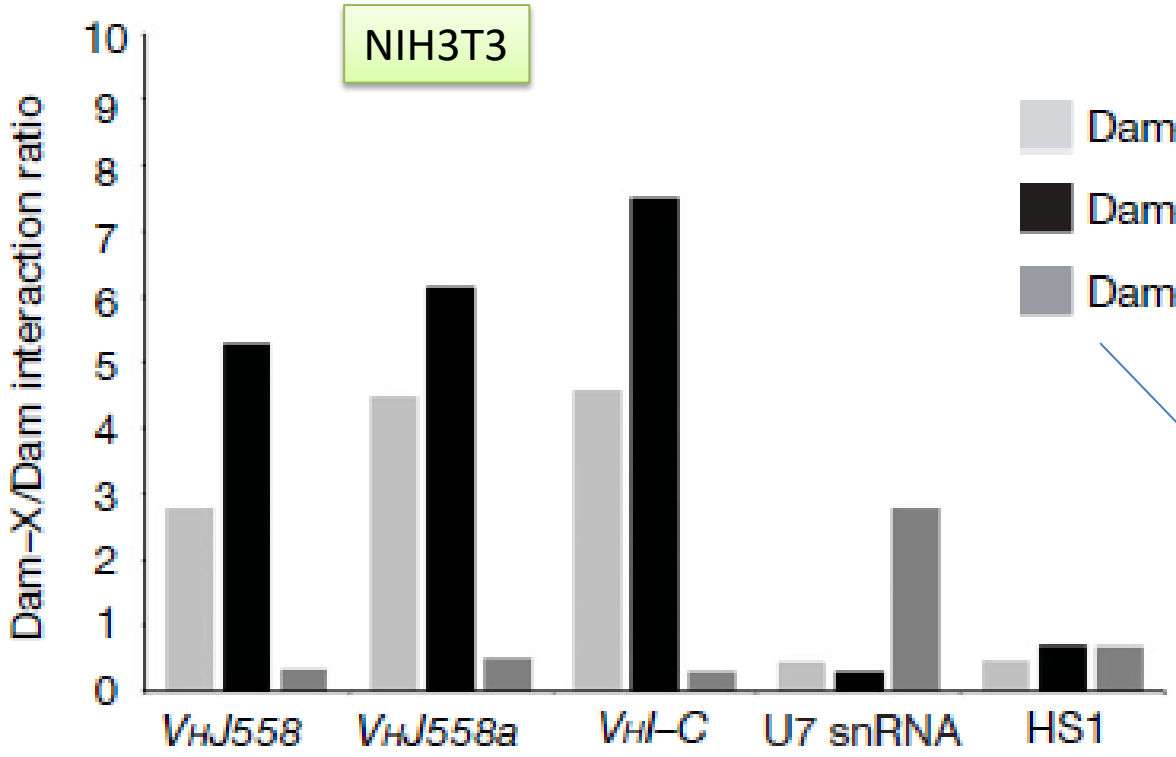


Figure 4 | Inactive immunoglobulin heavy chain loci that are positioned at the nuclear periphery contact the nuclear lamina. a, Interaction of Igh loci with the INM detected by 3D DNA-immunoFISH and DamID in NIH3T3 nuclei. The upper panel shows that a BAC probe hybridizing to the distal region of the Igh locus (green) colocalizes with the nuclear lamina (LMNB1, red).

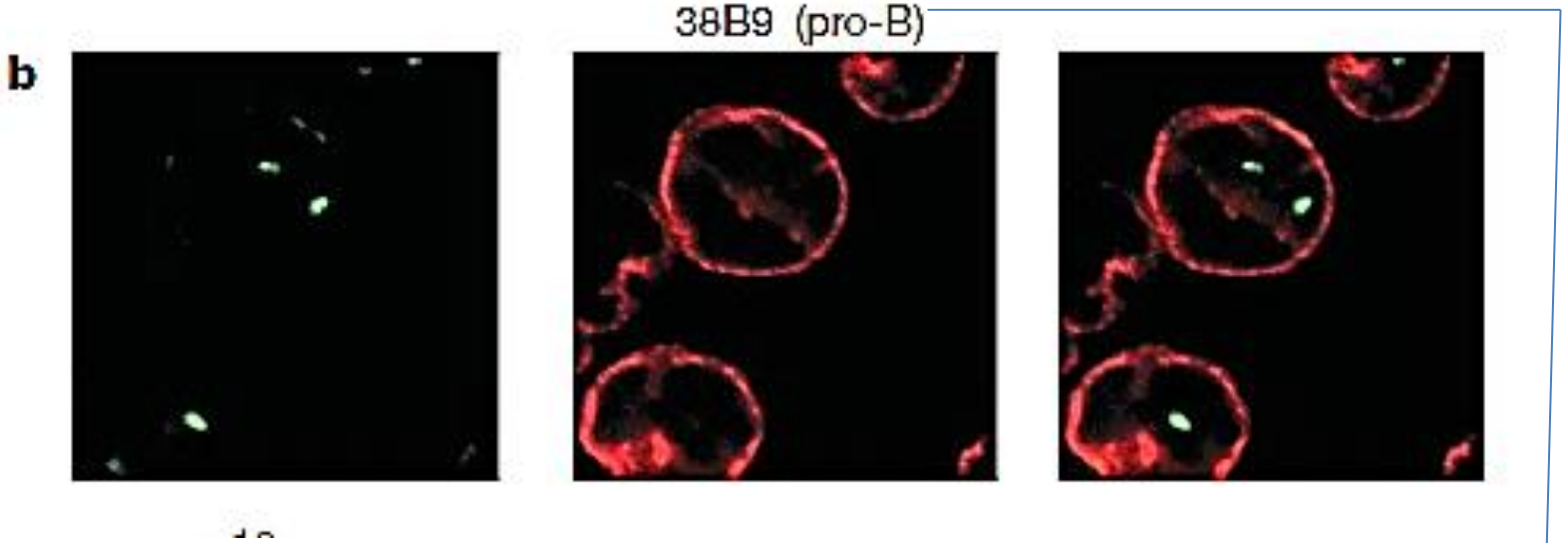


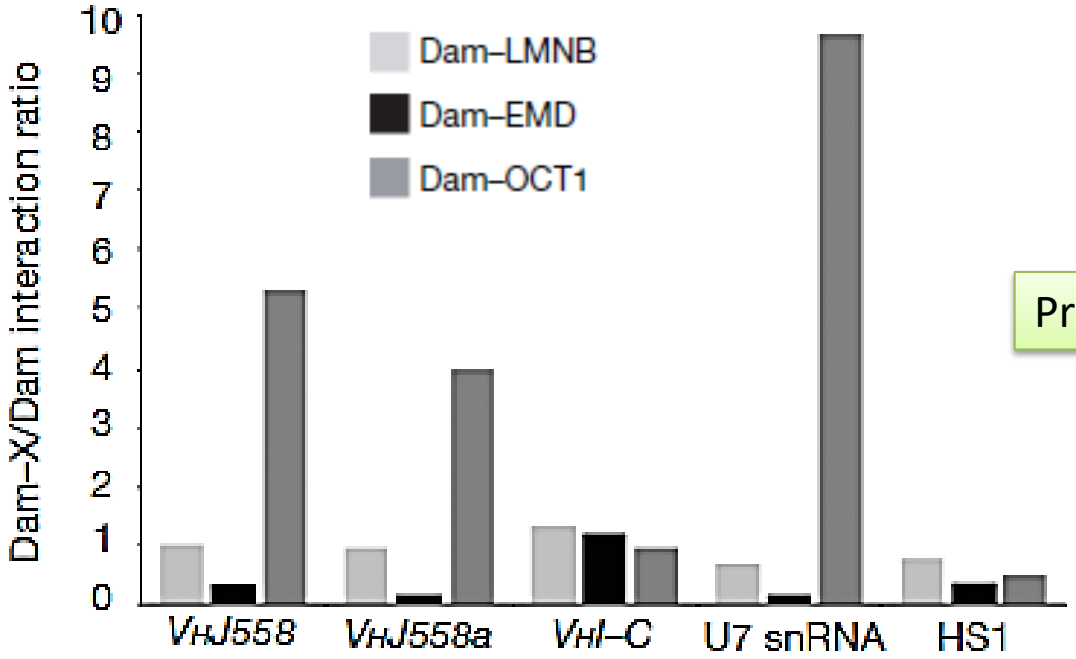
This checks for «accessibility»

The lower panel shows the molecular interactions of LMNB1 and EMD with the Igh loci in NIH3T3 nuclei detected by DamID-mediated methylation. Signals were normalized to a Dam-only control, and therefore the y-axis indicates the ratio of Dam–X (where X is LMNB1, EMD or OCT1) divided by the Dam-only signal. VHJ558 and VHJ558a correspond to promoter regions in the Igh VH region, whereas the VH intergenic region (VHI–C) is positioned 400 kb away from J558a. HS1 is a hypersensitive site immediately upstream of the most distal VHJ558 gene. The U7 snRNA genes are ubiquitously expressed and are regulated by OCT1.

This is fibrolasts, where the IgG locus is inactive

What about a B-cell line ?





The Igh locus is active in these cells

Pro-B cell line

with an endogenous locus. We suggest that, similar to our test gene, such interactions with the INM–lamina may establish an inactive state that inhibits access of transcriptional activators and the recombination machinery to *Igh* loci.

Conclusion, part 4

This is where the «discussion» start

How does attachment of a mammalian gene to the INM promote its repression? Two possibilities include, first, sequestration from the RNA Pol II apparatus and, second, assembly of a repressive chromatin structure.

245

Read the discussion carefully, it is important !