LETTERS

Transcriptional repression mediated by repositioning of genes to the nuclear lamina

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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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chromosomal segments bearing *lacO* sites. Interestingly, NPCs did not accumulate around the tethered chromatin domains and, in approximately half of the nuclei, NPCs were diminished at these regions (Fig. 2b). Furthermore, the tethered loci were not preferentially associated with pericentromeric heterochromatin in either clone-M or clone-S cells (Supplementary Fig. 5). Our results suggest that local accumulation of EMD by means of an association with chromatin can nucleate the assembly of specific INM and lamin components.

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

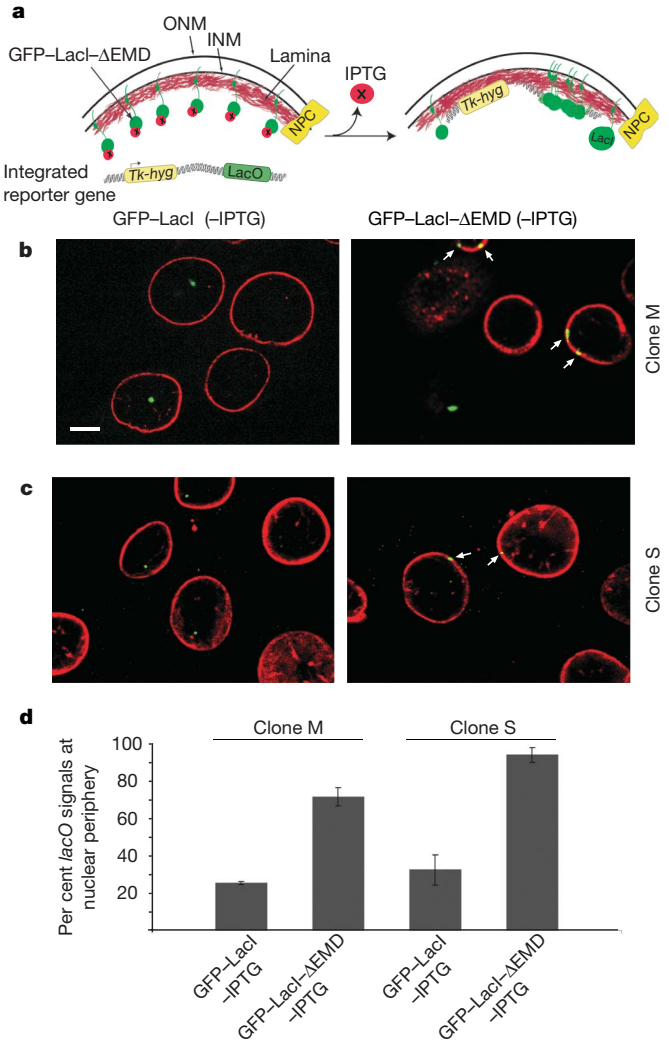

Figure 1 | Quantitative analysis of tethered loci by 3D DNA-immunoFISH. **a**, The stably integrated reporter gene contains an array of *lac* operators. The GFP–LacI–ΔEMD tethering protein is targeted to the INM and is unable to bind *lacO* sites in the presence of IPTG (red circles with cross in the 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. **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–ΔEMD 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. **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. **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.

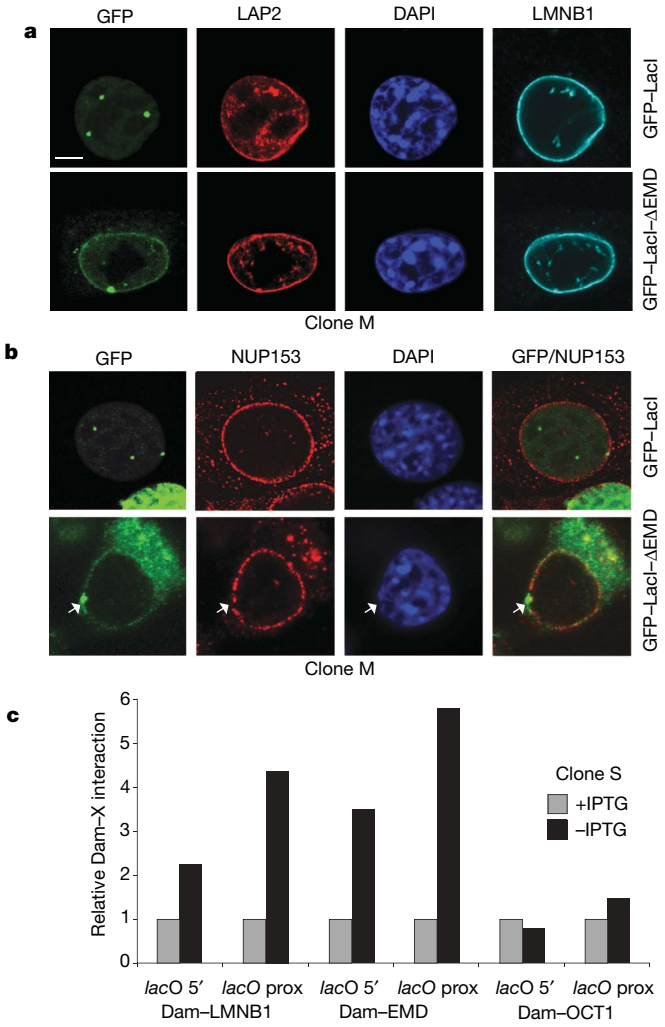

Figure 2 | Lamin B, EMD and LAP2, but not NPCs, accumulate at sites of tethered foci. **a**, **b**, Shown are *lacO* insertions detected by GFP fluorescence in clone-M cells expressing either GFP–LacI or GFP–LacI–ΔEMD upon IPTG withdrawal (24 h). Antibodies directed against LMNB1 (**a**, cyan), LAP2 (**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. **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 tranduced 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 (−IPTG). A representative experiment is shown. *lacO* 5′ and *lacO* prox are PCR primer pairs that are positioned upstream or downstream of the *lacO* arrays, respectively.

(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.

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 (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.

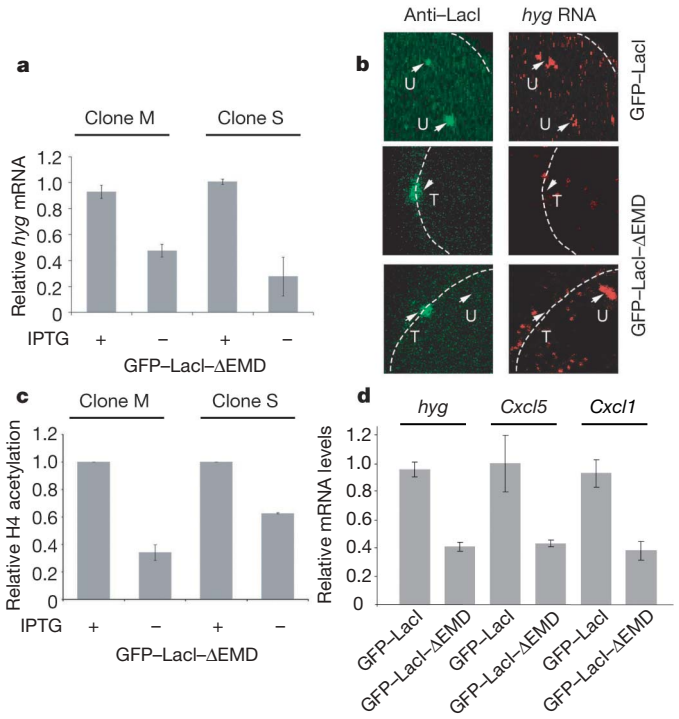

Figure 3 | Tethering leads to transcriptional repression and histone deacetylation of genes at site of attachment to the INM. **a**, Relative transcript levels of the *hyg* reporter gene were measured in clone-M and clone-S cells by Q-PCR under non-tethering (+IPTG) versus tethering (−IPTG) conditions. Transcript levels were normalized to endogenous mouse *Hprt1* messenger RNA. **b**, Three-dimensional RNA-immmunoFISH was performed on clone-M cells under non-tethering (GFP–LacI, −IPTG) versus tethering (GFP–LacI–ΔEMD, −IPTG) conditions. Foci detected by a LacI antibody (green) were scored for association with nascent *hyg* transcripts (red). Upper panels show untethered loci bound by GFP–LacI. The middle and lower panels show tethered (T) and untethered (U) foci in nuclei under tethering conditions. The dashed line indicates the edge of the nuclei, as determined by DAPI staining. **c**, Cross-linked chromatin from the indicated cells was immunoprecipitated using an antibody to acetylated histone H4. The histogram shows relative H4 acetylation levels of the HSV-TK promoter region of the test gene under the indicated conditions after normalization to the β-actin promoter. **d**, Q-PCR analysis of *hyg*, *Cxcl1* and *Cxcl5* mRNA in clone-M cells under the indicated conditions. Transcript levels were normalized to *Hprt1* mRNA. Error bars indicate the standard deviation between three experiments.

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.

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.

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 limited to the *Igh* gene promoters (*VHJ558* and *VHJ558a*), but were also detected in an intergenic region (*VHI–C*) of this *VH* gene family, suggesting multiple contacts with the INM and lamina over a genomic region spanning at least 400 kb (ref. 22). Importantly, no interaction was detected at a region upstream (HS1, 42 kb) of the *VHJ558* gene family[23]. Thus, the observed contacts of the *Igh* locus with the INM are not simply caused by cytological proximity, but probably reflect specific molecular interactions. Importantly, such INM–lamina contacts were not seen in pro-B nuclei (Fig. 4b). At this developmental stage, the *Igh* loci are transcriptionally active and positioned away from the nuclear periphery. As expected, OCT1 was seen to interact with the U7 small nuclear RNA gene promoter in both cell types. In contrast, OCT1 could access the *VH* gene promoters in pro-B but not in NIH3T3 nuclei. These findings demonstrate cell-type-specific molecular interactions of EMD and LMNB1 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.

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. In support of the former, the β-globin locus moves away from the nuclear periphery and engages with centrally positioned RNA Pol II factories to enable high levels of transcription[24]. Initial activation of this locus at the periphery may involve an association with NPC, as has been observed for yeast and *Drosophila* genes[9,10,25]. This mode of gene activation could also account for the behaviour of other mammalian genes that have been reported to be active while positioned at the nuclear periphery[4,26]. In support of the second possibility, we note that INM proteins interact with transcriptional co-repressors and chromatin-modifying enzymes[12–14]. Because tethering of a chromosomal region to the nuclear membrane by interactions with EMD can result in the accumulation of lamin A, lamin B and LAP2, we suggest that such self-reinforcing protein–DNA and protein–protein interactions could result in the establishment of a distinct repressive chromatin structure. We note the *lacO* arrays used in our test gene may poise it to undergo repression on association with the INM. Nevertheless, our results suggest the existence of DNA elements that function to repress gene activity by tethering chromosomal regions to the INM–lamina. Consistent with this possibility, genome-wide analysis in *Drosophila* cells has revealed clusters of transcriptionally silent and hypo-acetylated genes that interact with LMNB1 and are positioned at the nuclear periphery[20]. We propose that the INM–lamina compartment, which is a distinctive structural feature of metazoan nuclei, can be used to assemble repressive peripheral chromatin domains.

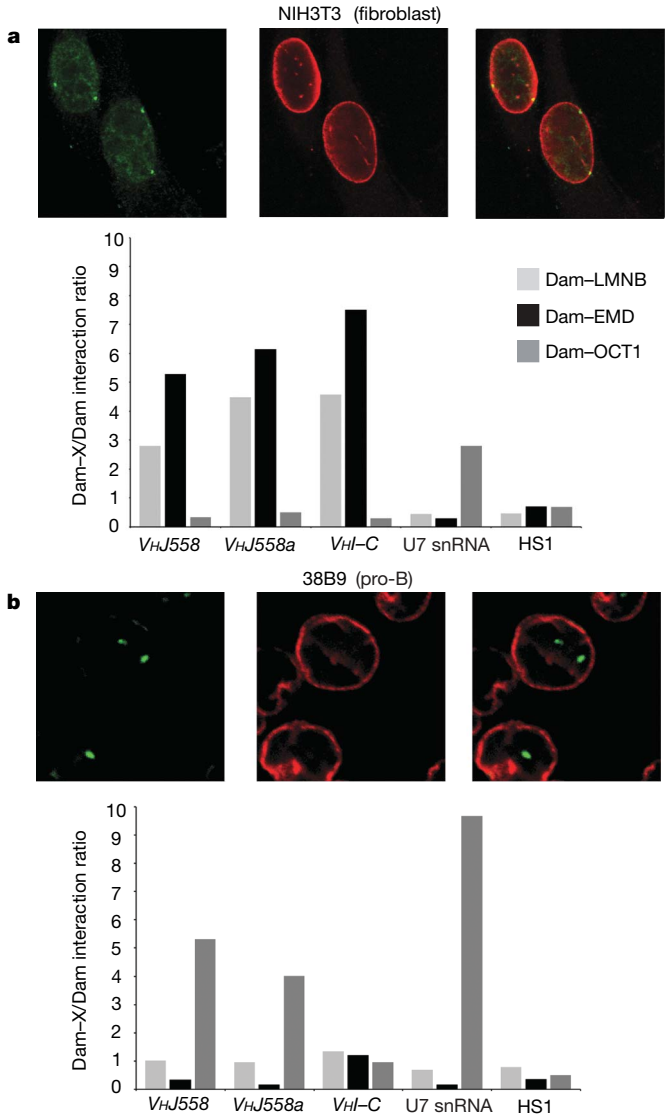

Figure 4 | Inactive immunoglobulin heavy chain loci that are positioned at the nuclear periphery contact the nuclear lamina. a, Interaction of *Igh* loci with the INM detected by 3D DNA-immunoFISH and DamID in NIH3T3 nuclei. The upper panel shows that a BAC probe hybridizing to the distal region of the *Igh* locus (green) colocalizes with the nuclear lamina (LMNB1, red). 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* V_H region, whereas the V_H 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. A representative experiment is shown. **b**, Interaction of the *Igh* loci with the INM detected by 3D DNA-immunoFISH and DamID in 38B9 pro-B-cell nuclei. In the upper panel, *Igh* loci and the nuclear lamina were detected by 3D DNA-immunoFISH as in **a**. The lower panel shows molecular interactions of OCT1, LMNB1 and EMD at *Igh* loci in 38B9 pro-B cells detected by DamID (normalized as above). In pro-B cells, V_H promoters are activated by OCT1.

METHODS SUMMARY

Tk-hyg lacO was constructed by modifying pSV2-DHFR 8.32 from A. Belmont[15]. The pMSCV(puro)eGFP–LacI retroviral vector was constructed using GFP–LacI from p3′ssEGFP–LacI. EGFP–LacI–ΔEMD and EGFP–LacI–ΔEMD* were generated by in-frame fusion with EGFP–LacI.

Immunofluorescence was performed using standard laboratory protocols[27]. Immunofluorescence coupled with 3D DNA-immunoFISH was performed as described previously[21]. Two-dimensional FISH procedures were performed as described previously[3]. Probes to the *lacO* sites and the *Cxcl5* genomic region (RP23-307P18) were generated by nick translation (Roche) with Alexafluor-568 (Molecular Probes) or biotin-labelled nucleotides. For RNA FISH, cells were plated, fixed and permeabilized as described previously[28]. All reagents were treated with DEPC to ensure RNase-free conditions. All DNA probes were labelled by nick translation (Roche) with Alexafluor-568 (Invitrogen), biotin or digoxigenin (Roche) nucleotides. Protein-antigen detection was performed as described for immunofluorescence. Images were obtained by laser scanning confocal microscopy using a Leica SP2 AOBS (University of Chicago, Digital Light Microscopy Facility) and an Olympus Fluoview 1000. For quantitative image analysis, 3D DNA-immunoFISH samples were imaged at ×100 using a ×2 optical zoom, with a *z*-step of 0.12 μm between optical slices.

RNA was prepared using TRIzol reagent (Invitrogen), and complementary DNA was generated using SuperScriptII reverse transcriptase (Invitrogen). Q-PCR experiments were performed on a Stratagene Mx4000 machine using SYBR green dye master mix (Stratagene). All reactions were compared to a standard curve to enable determination of the relative copy number.

For microarray analysis, RNA was prepared using TRIzol reagent. Probe preparation, hybridization and initial analyses were carried out by the Functional Genomics Facility at the University of Chicago. Two or more genes within 500 kb were scored as a cluster.

DamID was performed as described previously using self-inactivating retroviral vectors for transduction[21].

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions K.L.R. designed and performed most of the experiments. J.M.Z. carried out the DamID experiments with K.L.R.. E.B. contributed the microarray analysis. K.L.R. and H.S. wrote the manuscript.

Author Information The microarray data can be found at the Gene Expression Omnibus at NCBI (http://www.ncbi.nlm.nih.gov/projects/geo/) under accession number GSE10176. Reprints and permissions information is available at www.nature.com/reprints. Correspondence and requests for materials should be addressed to H.S. (hsingh@uchicago.edu).