Subnuclear localisation is associated with gene expression more than parental origin at the imprinted \textit{Dlk1-Dio3} locus

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Abstract

At interphase, de-condensed chromosomes have a non-random three-dimensional architecture within the nucleus, however, little is known about the extent to which nuclear organisation might influence expression or vice versa. Here, using imprinting as a model, we use 3D RNA- and DNA-fluorescence-in-situ-hybridisation in normal and mutant mouse embryonic stem cell lines to assess the relationship between imprinting control, gene expression and allelic distance from the nuclear periphery. We compared the two parentally inherited imprinted domains at the \textit{Dlk1-Dio3} domain and find a small but reproducible trend for the maternally inherited domain to be further away from the periphery however we did not observe an enrichment of inactive alleles in the immediate vicinity of the nuclear envelope. Using Zfp57KO ES cells, which harbour a paternal to maternal epigenotype switch, we observe that expressed alleles are significantly further away from the nuclear periphery. However, within individual nuclei, alleles closer to the periphery are equally likely to be expressed as those further away. In other words, absolute position does not predict expression. Taken together, this suggests that whilst stochastic activation can cause subtle shifts in localisation for this locus, there is no dramatic relocation of alleles upon gene activation. Our results suggest that transcriptional activity, rather than the parent-of-origin, defines subnuclear localisation at an endogenous imprinted domain.

Author summary

Genomic imprinting is an epigenetically regulated process that results in the preferential expression of a subset of developmentally regulated genes from either the maternally or the paternally inherited chromosomes. We have used imprinted genes as a model system to investigate the relationship between the parental origin of the chromosomes, the localisation of genes within the cell nucleus and their active expression. By assessing the location of the \textit{Dlk1-Dio3} region and the expression of the \textit{Gtl2/Meg3} gene within it, we find that there is a small preference for the paternal chromosome to be closer to the periphery.
but a significant correlation between transcription and distance to the edge of the nucleus for the \textit{Gtl2/Meg3} gene. Furthermore, a chromosome nearer the periphery is just as likely to express the gene as the chromosome further away. These findings suggest that the parental origin of the chromosome may not be as important as the transcription of the gene in defining the position of the imprinted region within the nucleus.

**Introduction**

The spatial organization of chromosomes in the interphase nucleus is non-random and involves 3D interactions on chromatin as well as interactions with various nuclear domains, the nuclear envelope being the best characterized \cite{1}. While the bulk of chromosomes is arranged as relatively compact and spatially defined chromosome territories \cite{2}, open chromatin between these contains transcription factories where active genes from different genomic loci co-localize at times of transcription \cite{3}. Recently, there has been much interest in understanding ways in which the 3D localisation of chromatin can influence or be influenced by gene expression \cite{4,5}. While chromatin organization into chromosome territories, topologically associated domains, and loops have complex effects on transcriptional regulation, interactions with the nuclear envelope are generally seen as transcriptionally repressive \cite{1}. Lamina associated domains (LADs) have been characterized as gene poor, transcriptionally inactive regions \cite{6}, even though these repressed regions have been shown to be dynamic in dividing cells and are therefore not always lamina-associated in every cell within a population \cite{7}. A notable exception to the repressive environment at the nuclear periphery is represented by nuclear pore complexes, where chromatin-facing nucleoporins have been implicated in transcriptional activation rather than repression in yeast \cite{8}. However, in Drosophila, nucleoporins appear to have this activating effect mainly in the nuclear interior \cite{9–11}. In line with the general notion that the nuclear periphery acts as a repressive environment, some though not all individual genomic loci have been shown to become transcriptionally repressed after artificial targeting to the nuclear lamina \cite{12–17}. These findings suggest a role for subnuclear position in gene regulation, however, the extent and importance of non-random localisation is poorly understood.

The \textit{Dlk1-Dio3} imprinted domain on mouse chromosome 12 is well characterized and is a valuable model for comparative analysis of gene regulatory mechanisms. Impprinted genomic regions contain genes that are preferentially expressed from either the maternally or paternally inherited chromosome, but that also are subject to the same regulated and stochastic transcriptional mechanisms that govern the expression of other genes. Impprinted genes are therefore regulated by both germline-derived parental-origin-specific epigenetic mechanisms and the transcriptional milieu of a particular cell type \cite{18,19}. \textit{Dlk1-Dio3} imprinting (Fig 1A) is regulated by an intergenic differentially methylated region (IGDMR) and contains a number of paternally expressed protein-coding genes as well as maternally expressed non-coding RNAs. Recent evidence has explored the relationship between the parental origin of this imprinted domain and its subnuclear locations. Kota and colleagues identified differential localisation of the \textit{Gtl2/Meg3} gene in a mouse embryonal stem (ES) cell line with the non-expressed paternal copy being closer to the nuclear periphery than the expressed maternal allele \cite{20}. Furthermore, a LINE1 (L1) repeat cluster located between \textit{Begain} and \textit{Dlk1} within the cluster (Fig 1A) was described to represent a facultative LAD in mouse ES cell-derived neural stem cells \cite{21,22}. Although this suggests that the L1 repeat might have an inhibiting effect on local gene expression via lamina tethering, deletion of the repeat cluster itself did not lead to increased
expression of neighbouring genes [21]. Here, we use extensive 3D RNA-DNA fluorescence-in-situ-hybridisation (FISH) in multiple normal and mutant mouse ES cell lines and show that gene expression state rather than the parental origin of alleles is associated with differences in intranuclear localisation, and suggest that juxtaposition to the nuclear envelope appears to play a minor role, if any, in gene regulation at this endogenous locus.
Results
Differential intranuclear distribution of parental alleles of the Dlk1-Dio3 region

In order to analyse the intranuclear localisation of alleles of the Dlk1-Dio3 region, while knowing their parental origin, we generated ES cell lines derived from delL1rep maternal and paternal heterozygous mice [21]. We used only male ES cell lines for this study since it has been previously shown that imprinting status is more stable in male ES cells compared to female ES cells [23]. These cells carry a deletion between the genes Begain and Dlk1, which includes a 170kb L1 repeat array (Figs 1A and S1) and have a normal epigenotype. Using a DNA FISH probe specific to the L1 repeat array, we used the deletion to distinguish the two parental chromosomes in 3D DNA FISH experiments, independent of gene expression. Importantly, we quantified the behaviour of WT chromosomes not carrying the deletion. For each cell we measured the 3D distance of the DNA FISH probe to the nuclear periphery defined by the edge of DAPI staining in an automated way (Fig 1C). To ensure that DAPI staining faithfully captured the position of the lamina, using a separate set of ES cells (n = 84), we compared edge measurements taken by DAPI staining compared to staining of the lamin in the same cells. Whilst lamin staining results in two visible borders (an outer and an inner) both measurements were highly correlated, suggesting that DAPI provides a reasonable estimate of the relative distance relationships between cell lines (t = 94.309, p < 2e-16, estimate (slope) = 0.993 and t = 138.12, p < 2e-16, estimate (slope) = 0.996 for inner and outer lamin border, respectively; S2 Fig). Therefore, the DAPI edge measurement was used as a read-out for the intranuclear position of the Dlk-Dio3 imprinted region, which lies within a single TAD in ES cells (S1 Fig) [24,25]. In parallel, we used nascent RNA FISH to obtain the Gtl2/Meg3 expression state (expressed or non-expressed) for each allele.

We took DNA FISH probe distance measurements in three biological replicates each of heterozygous ES cells derived from maternal inheritance of delL1rep (matrepKO), paternal inheritance of delL1rep (patrepKO) and WT ES cells, this way comparing paternally inherited alleles, maternally inherited alleles and the mixture of both as a control. Using a multiple least-squares linear regression model, we find that paternal alleles are significantly closer to the nuclear periphery compared to maternal alleles after controlling for nuclear volume (t = 2.946, p = 0.00334, estimate (slope) = 0.22 μm; Fig 2A). Given that WT cells contained both alleles, we decided to characterise the relationship between the two alleles to understand their natural distribution relative to each other within these cells. In WT cells, one allele was always closer to the nuclear border than the other, and there was a significant difference between the two alleles (t = 22.80, p < 2e-16, estimate (slope) = 1.08 μm; Fig 2A). This is consistent with a second set of WT cells that were used in this study (WT2; t = 22.59, p < 2e-16, estimate (slope) = 0.99 μm). While the parental origin of the WT alleles was unknown, we assume that this difference might, at least in part, reflect the tendency for the paternal allele to be biased towards the periphery, as demonstrated in previous reports [20]. However, in the KO cells, where the accuracy of parental origin is ensured, the average difference in distance between the maternal (M = 1.81 μm, SD = 1.00 μm) and the paternal allele (M = 1.58 μm, SD = 0.954 μm) was unexpectedly small (absolute raw mean difference of ~230nm). Previous work from Kota and colleagues had found differences of more than a micrometer [20], a difference that is consistent to that between the WT near and far alleles regardless of parental origin, but not the maternal and paternal alleles in this study (Fig 2A). This small difference that we observed in KO cells was not due to variability in replicates as replicates were not significantly different from one another (S3 Fig), but suggested that the parental origin effect we observed might be of limited
functional significance. We, therefore, developed a genetic approach to compare the two parental chromosomes in a more functional context.

Epigenotype switching does not shift localisation

To address the relationship between parent of origin and function more overtly we utilized genetic models exhibiting an epigenotype switch that reversed the imprints on either the maternal or the paternal chromosome. We use the term ‘epigenotype’ to refer to the parental-origin specific epigenetic marks (both histone modifications and DNA methylation) that distinguish the two parental chromosomes. For example, the paternal and maternal IG-DMR are enriched with H3K9me3 and H3K4me3/H3K27ac, respectively [19,26–28]. The maternally inherited \textit{Gtl2} promoter also exhibits substantial parental origin specific differential DNA methylation—the paternal allele is hypermethylated, the maternal allele is hypomethylated [19,28].

\textit{Zfp57KO} ES cells carry a null mutation in the chromatin modifier \textit{ZFP57}, which is important for imprint maintenance and, in the case of the \textit{Dlk1-Dio3} region, results in a paternal to maternal epigenotype switch causing a maternal expression pattern from the paternally inherited chromosome (Figs 1A and 1B and S4) [26,29] Conversely, matIGDMRKO ES cells are derived from blastocysts that have a maternally inherited deletion of the IGDMR, resulting in a maternal to paternal epigenotype switch and expression of the paternally expressed imprinted genes from the maternally inherited chromosome [30]. Therefore, in matIGDMRKO and \textit{Zfp57KO} lines, both copies of the imprinted domain now behave (\textit{i.e.}, are expressed) as if they were paternal or maternal copies, respectively. For this experiment, we generated additional matched WT control ES cells (WT2) using WT littermates of KO blastocysts as before (see Methods). Using three biological replicates for each mutant and control line, we found that there was no significant difference in the subnuclear localisation of mutant chromosomes compared to WT chromosomes between cell types ($t = 0.143$, $p = n.s$; Fig 2B). This was true, even after effects of nuclear volume were taken into account. These findings indicate that maternalising the paternal domain and paternalising the maternal domain, do not result in the expected shift in localisation and therefore indicate that epigenotype is not...
determining the localisation. However, these data do not consider the current transcriptional state of imprinted loci in these models.

**Stochastic or developmental repression of the maternally expressed Gtl2/Meg3 gene correlates with a shift of alleles towards the nuclear periphery in ES cells**

While Dlk1 is not expressed in ES cells, Gtl2/Meg3 is strongly expressed. Though all maternally inherited alleles of the Gtl2/Meg3 gene have the potential to express the gene, it is not active in all cells (S5 Fig). It is not known whether this heterogeneity in expression is stochastic or regulated. We know that, gene expression can be fine-tuned by transcription on-off cycles, sometimes called “bursting”, which could reflect genes moving in and out of transcription factories [3]. Alternatively, some individual ES cells may change their properties resulting in changes in gene expression that could include Meg3 (i.e., through differentiation). Since we cannot distinguish these possible types of repression in our experiments, we refer to Gtl2/Meg3-non-expressing maternal alleles as “stochastic or developmental repression”. Stochastic or developmental repression of the canonically active maternal allele of Gtl2/Meg3 was observed in 29.5% of WT ES cells and 32.3% of patRepKO ES cells (S5 Fig). We took advantage of this property to test whether Gtl2/Meg3 alleles differed in their intranuclear localisation (i.e., distance from L1 to the nuclear envelope) based on their expression state as measured by RNA FISH (for the active alleles). We used a logistic regression approach to test if distance to the periphery predicted the expression state of Gtl2/Meg3 (after controlling for nuclear volume variation). In three biological replicates each of patrepKO ES cell lines in which the single maternally inherited Meg3 allele has the potential to be either expressed or not, we found no difference in localisation regardless of the expression of Gtl2/Meg3 (z = 1.059, p = n.s; Fig 3A).

However, since Zfp57KO ES cells have twice as many maternal(ized) alleles as patrepKO ES cells, we reasoned that experiments with these cell lines would have greater power to examine the relationship between distance and expression. Using the same statistical methodology and same numbers of replicates, we found a non-significant trend (estimate = 0.927μm, p = 0.0721, Fig 3B) with non-expressed alleles being marginally closer to the periphery compared to expressed alleles. As before, nuclear volume was taken into account. Together these findings indicate insignificant effects of imprinted repression (i.e., epigenetic silencing) on localisation relative to the nuclear periphery. We, therefore, focused on maternalised expressed alleles to consider whether stochastic expression states rather than imprinted repression might better predict localisation.

**Gene expression predicts localisation better than parental epigenotype**

Using Zfp57KO cells, we can measure whether biallelic expression could predict distance from the nuclear border better than monoallelic expression or no expression from either allele. Using a multiple linear mixed model approach we find that there is a marginal increase in distance when only one allele within the cell is expressed (t = 1.837, p = 0.067, estimate (slope) = 0.175 μm) but alleles move significantly further from the nuclear border when both alleles are expressed (t = 1.989, p < 0.05, estimate (slope) = 0.185 μm; Fig 3C). This suggests that taking into account transcriptional state predicts a more centralised localisation. This could be due to gene dosage or reflect the overall increase in transcriptional activity of two active alleles within a cell. Nevertheless, it suggests that activation is correlated with a shift away from the nuclear border. This is consistent with the idea that maternalised repressed alleles are more similar in localisation to paternal alleles (epigenetically silenced) than paternal alleles that have escaped imprinting (S6 Fig).
We further explored the relationship between localisation and expression by examining whether alleles classified as ‘near’ and ‘far’ from the nuclear border were more or less likely to be expressed based on their relative position. We find no such relationship in WT (log$_2$(OR) = 0.95, p = 0.80; Fishers Test) or Zfp57KO cells (log$_2$(OR) = 0.93, p = 0.77; Fishers Test; Fig 4A) suggesting that both near and far alleles have an equal probability of being expressed. This does not consider distance from the periphery but rather the relative position of the two alleles within the nucleus. Hence, this makes no claims about the function of the nuclear periphery since it is possible that both maternal and paternal alleles in this model are too far from the periphery to undergo lamina-associated repression. Consistent with this and as shown in Fig 4B, repressed maternalised alleles (patrepKO and Zfp57KO combined here) are not more likely to be greater than 0.5µm away from the periphery ($\chi^2 = 0.10$, df = 1, p = n.s). To examine this at higher resolution, we plotted the regional location after dividing the nucleus into thirds by volume (outer, middle, inner) as has been described previously [20]. Results suggest that stochastically repressed alleles are not preferentially located to the periphery ($\chi^2 = 2.3834$, df = 2, p = n.s; Fig 4B). A similar pattern was found in WT cells (see S7 Fig), indicating that there is no distinct areas of localisation between expressed and transcriptionally silent alleles.

**Discussion**

We performed 3D RNA-DNA FISH measurements in a total of 18 mouse ES cell lines and observed a very small but overall highly significant effect for Gtl2/Meg3-expressing alleles of the Dlk1-Dio3 imprinted region to be localized towards the nuclear interior and for non-expressing alleles to be localized towards the nuclear periphery. As has been shown before [20], paternal alleles, which do not express Gtl2/Meg3, localize overall closer to the periphery than maternal alleles, from which Gtl2/Meg3 is expressed in 70.5% of WT ES cells. Differences
in localisation between paternalized and maternalized alleles (i.e., IGDMRKO vs ZFP57KO) are only evident when expression state is considered. In other words, expressed Gtl2/Meg3 alleles are positioned further away from the periphery than non-expressed alleles. This idea is consistent with previous work showing that the tethering of transcriptional activators to a peripheral chromosome site can lead to movement of the chromosome site towards the nuclear interior [31,32]. Furthermore, the position within the nucleus is directly related to the number of alleles being expressed within a given cell, as evident through the analysis of cells expressing zero, one or two alleles. Future studies can address the biological relevance, if any, of this intriguing finding.

The effect size of the localisation difference between expressing and non-expressing alleles in our study is much smaller than that previously described (19). Three notable differences

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Fig 4. Gtl2/Meg3-non-expressing maternalized alleles are not enriched at the nuclear periphery. (A) Being the near vs far allele did not affect the likelihood of being expressed in either wild-type (WT) or Zfp57KO ES cells. (B) The majority of maternal (patrepKO) and maternalised alleles (Zfp57KO) are localised away from the periphery with no difference between Gtl2/Meg3 expressing and non-expressing alleles (top). Nor were there any significant differences in distribution of expressed and non-expressed alleles when the nucleus was divided into three equal volume bins (an inner, middle and outer; bottom).

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between our work and the previous study might explain this: 1) Kota et al. [33] used *Gtl2/Meg3* expression as a read-out for parental origin while we included an independent DNA marker and genetic approaches allowing us to distinguish and quantify expressing and non-expressing maternal chromosomes. 2) Kota et al. [33] used fewer biological replicates hence would not have been able to take into account the variability that is evident between lines. 3) Our measurements utilise a DNA marker located 400kb away from the interrogated gene which might result in lower data resolution (S1 Fig). Both genetic locations however, lie within the same TAD in ES cells [24,25] and show frequent overlap in a double probe DNA FISH experiment using probes against the L1 repeat and the *Gtl2/Meg3* gene (S1 and S8 Figs). However, these locations do lie in different sub-TADs which may partially explain discrepancies between our results and Kota et al. [33]. Our findings reflect the importance of independently distinguishing the parental chromosomes themselves from their expression, and then determining the contribution that each makes to subnuclear localisation.

Mouse ES cells show an unusual cell cycle distribution where a majority of cells (75%) will be in S-phase in a growing cell population [34]. S-phase can potentially have an influence on gene expression and cell cycle heterogeneity could thus be a source of variability between biological replicates. However, when comparing nuclear sizes (as a proxy for cell-cycle differences) of expressing and non-expressing alleles as well as biological replicates, some small variation in nuclear size was evident in some instances (S9 Fig). We therefore included nuclear volume as a covariate in all of our analyses to correct for any minor fluctuations in nuclear volume. Hence, all of our estimates of the differences in allelic distribution control for minor changes in nuclear volume.

Though data from all our ES cell lines show a significant expression-dependent effect on subnuclear localisation, the effect size is extremely small with *Gtl2/Meg3*-non-expressing alleles being on average only 170 nm closer to the nuclear periphery than *Gtl2/Meg3*-expressing alleles. Indeed, most expressed alleles can be more broadly categorised as being away from the periphery (or outer most region) though not extremely central to the nucleus. It is interesting to consider whether such a small effect is biologically meaningful. Since we did not observe a significant enrichment of inactive alleles in the immediate vicinity of the nuclear envelope, inactivating interactions with the nuclear lamina are unlikely to play a major functional role at this imprinted domain. Notably, *Gtl2/Meg3* expression was regularly found right at the edge of DAPI staining probably representing areas of low-density chromatin at the very periphery of nuclei (see methods section for details). Although it can be envisioned that in some cases *Gtl2/Meg3* expression might have been inhibited by transient nuclear envelope interactions and had already moved away from the periphery at the time of the experiment, such alleles should still show up as peripheral enrichment since mobility of individual loci during interphase is usually limited to about 1 μm and it has been shown that large scale changes in localization require cell division [1,35].

A more likely explanation is that the observed pattern of allele localisation is due to some activating effect of structures at the nuclear interior. This is consistent with the increase in internalisation observed when two alleles are expressed compared to one in a given cell. Transcription is known to happen preferentially at sites where the transcriptional machinery and active genes are clustered into so-called transcription factories and adjacent sites known as speckles [3,4,32]. Such factories are found in open chromatin at the borders of chromosomal territories and it seems logical that they would be biased to be more frequent at the more transcriptionally favourable nuclear interior. Therefore, one possible explanation for the observed non-enrichment at the nuclear periphery and the small effect size of our expression-localisation correlation could be that *Gtl2/Meg3*-non-expressing alleles are evenly distributed within the space they can take up around the chromosome territory, independent of nuclear envelope
interactions, while Gtl2/Meg3-expressing alleles need to be localized at transcription factories which are biased to be at the interior. It would be interesting to test this idea by co-localising expressing and non-expressing alleles with components of transcriptional factories. Nevertheless, our use of multiple biological replicates in multiple genetic models highlights the wide range of positions that an allele can take within the nucleus regardless of expression status.

For all correlations of nuclear localisation and expression, the question of cause and consequence arises. Is peripheral localisation used as a means to fine-tune gene expression by making the co-localisation with transcription factories more or less likely? The absence of enrichment of inactive alleles at the nuclear periphery does not argue for such a mechanism. Kota and colleagues have shown that position changes of the Dlk1-Dio3 region are local and do not involve gross changes in chromosome territory [20]. Similarly, our Zfp57 mutants show that cis acting epigenetic changes relate to a shift in localisation at the same time as inducing Gtl2/Meg3 gene expression. This argues that a nuclear envelope anchoring mechanism acting at a distance is unlikely to be involved. A local anchoring mechanism should show up as peripheral enrichment and we do not observe this. We therefore suggest that the small shift in peripheral localisation that we observe is a consequence, rather than a cause of expression changes and that the more functionally relevant aspect is the interior location of expressed alleles.

Methods

Cell culture

Mutant and corresponding control ES cells were generated from single blastocyst using feeder-free-based 2i LIF culture conditions (N2B27, Stem Cell Sciences) [36]. In brief, morula embryos were collected from pregnant female mice and cultured in KSOM with 2i inhibitors, and then each blastocyst was genotyped using trophoderm after immunosurgery. delL1rep mice have an albino C57BL/6J background (BL6) [21] and were crossed to albino BL6 mice to obtain WT, maternal and paternal heterozygotes. IG-DMR heterozygous females [30] were mated with BL6 males to obtain IG-DMR maternal KO and control WT morula embryos. Zfp57 zygotic KO ES cells and corresponding control ES cells were generated in a previous study [26]. We observed unexpectedly low expression levels of Gtl2/Meg3 in two of five Zfp57KOES cell lines and therefore excluded these two cell lines from these studies. For our analysis of ES cells of all genotypes, we used data from the three biological replicates that showed the expected and similar proportions of non-expressing, monoallelically expressing, and biallelically expressing cells (S5 Fig). This research was approved by the Animal Welfare and Ethical Review Body (AWERB) of the University of Cambridge and conducted in accordance with UK Home Office Animals Scientific Procedures Act under project licence 80/2567.

Fluorescence in situ hybridisation

We generated fluorescent probes for detection of the LINE repeat (BAC bMQ-177C10, obtained from CHORI) and nascent Gtl2/Meg3 transcripts (fosmid WIBR1-2686H19, a kind gift from the Heard lab, Paris) by first amplifying plasmid mini preps with the Illustra Templi-Phi Large Construct Kit (GE Healthcare) and subsequently labelling 2 μg of the amplification product by nick translation using Green UTP or Red UTP (Abbott Molecular) according to the manufacturer’s instructions. Sequential RNA and DNA 3D fluorescence in situ hybridisation (FISH) was performed as previously described [37]. However, ES cells were grown on laminin-coated coverslips prior to fixing to ensure well-spread monolayer growth and nuclei were counterstained with 0.2 μg/ml DAPI after hybridisation and washes. 3D image stacks of 10–15 positions per coverslip were acquired using a Carl Zeiss Axiovert 200M microscope.
with a 63x/1.25 Plan Apochromat objective taking 100 image planes per image stack at a z spacing of 0.15 μm. For double probe DNA FISH the same protocol was used as in sequential FISH and the Gtl2/Meg3 fosmid probe was used to detect the gene rather than nascent RNA.

**Image analysis**

DNA FISH image stacks were post-processed using Huygens Professional deconvolution software (version 14.10.1p8, SVI, The Netherlands) and 3D measurements were taken using Fiji [38]. We developed a Fiji script that enabled us to generate binary representations of DAPI-stained nuclei and FISH signals and to take automated 3D measurements of and between these objects calling Fiji’s 3D manager function (Fig 1C). If FISH signals were duplicated due to DNA replication, we used the mean of both measurements for the analysis. Due to the nature of the binarization process using DAPI staining of chromatin, binary images of nuclei sometimes differed slightly in shape from what was seen by eye in the original image because, depending on image background levels or closeness of neighbouring nuclei, non-dense chromatin regions were interpreted as background by the algorithm. This led to a minority of DNA FISH signals (2.8%) being outside the nuclear volume. To avoid bias against peripherally located signals, we included such signals as negative distance measurements. In very rare cases the algorithm generated a bay-like background area around a FISH signal in a non-dense chromatin area, in a way that our standard automated measurements would lead to false results. Again, to avoid biasing against peripheral signals as well as inaccurate manual measuring, in these rare cases (0.3%) we used an alternative algorithm that approximates the nuclear volume by fitting an ellipse around the original shape and used this shape for distance measurements. For relative distance measurements, we used the “radiusCen” measurement of Fiji’s 3D manager, which measured the distance between the centre of the binary representation of the nucleus and its border through the centre of the binary representation of the FISH signal, as local nuclear radius. Relative distances were calculated as absolute distance / local nuclear radius. In parallel to DNA FISH analysis, the expression state of the Gtl2/Meg3 gene was evaluated from original (non-deconvolved) nascent RNA FISH image stacks for each allele as either expressed (FISH signal above background level) or non-expressed (no signal).

**Lamin staining by immunofluorescence**

E14tg2a ES cells were cultured on laminin-coated coverslips, fixed for 10 minutes in 3% paraformaldehyde and permeabilized in 0.5% Triton X-100. Cells were then blocked in 1% BSA for 15 minutes and then incubated in 1:200 Goat α Lamin B1 (Santa Cruz SC-30264) 1% BSA for 45 minutes at room temperature. The secondary antibody, 1:500 Donkey α goat IgG Alexa 633 1% BSA, was applied and cells incubated for 40 minutes at room temperature in a dark humid chamber. The cells were then counterstained for 1 minute in 1X PBS containing 0.2mg/ml DAPI and mounted in mounting medium. 3D image stacks were acquired (see Fluorescence in situ hybridisation methods) and image stacks were post-processed using Huygens Professional deconvolution 338 software (version 14.10.1p8, SVI, The Netherlands). Distances were measured using Fiji.

**Statistical methodology**

All analyses used a linear regression model framework using nuclear size as a covariate (to control for any volume effects on expression and/or localisation) using base R regression functions [39]. Given that some cells had measurements for multiple alleles, where appropriate, we used mixed effects multiple linear regression models using the lme4 and lmerTest packages in R [40,41] to allow for random intercepts (of unique cells) which effectively controls for variability.
arising from the repeated measurement of unique cells [42]. Data handling and visualisation in R was carried out using the ‘tidyverse’ packages [43]. Chi-squared tests for differences in nuclear volume distributions were calculated using base chi.sq functions in R. Fishers’ tests of enrichment were performed using the base R fisher.test function.

Supporting information

S1 Fig. UCSC (https://genome.ucsc.edu/) -screenshot of the Dlk1-Dio3 imprinted region in mouse mm9 showing the exact positions of RNA (Meg3) and DNA (L1 repeat) FISH probes, as well as the genomic distances between them (inner, central and outer distance). The exact position of the LINE1 repeat deletion [21] in relation to the repeat probe and the UCSC RepeatMasker are also shown. The top panel shows ES cell TADs from Schoenfelder and colleagues (Bab_ESC) and Dixon and colleagues (Ren_ESC) loaded as custom tracks [24,25] (TIFF)

S2 Fig. Correlation plots of DAPI edge measurements (μm) compared to lamin inner (a) and outer (b) borders (μm).

(TIFF)

S3 Fig. Plots of the distance from the nuclear border relative to Gtl2/Meg3 expression in individual biological replicates of all KO and WT groups measured in the study. We find no significant effect of replicate on overall distance measures.

(TIFF)

S4 Fig. Additional examples of gene expression in the five ES cells genotypes at larger magnifications (compare Fig 1). Gtl2/Meg3 probe stained images are from nascent RNA FISH and L1 Repeat probe stained images are from subsequent DNA FISH. The FISH images represent maximum projections of 10–30 central z planes of acquired image stacks. Blow-ups of framed regions are shown on the right. Scale bars represent 5 μm.

(TIFF)

S5 Fig. Numbers of cells per cell line with no, monoallelic, or biallelic Gtl2/Meg3 expression.

(TIFF)

S6 Fig. A) Distribution of alleles split by genotype and expression-type. Non-expressed maternal and paternalised alleles look similar in distribution to unexpressed paternal and paternalised alleles (not significantly different). Activated alleles (either through imprinting escape or normal maternalised expression) are generally further away from the nuclear border. We did formally test the effect of imprinting escape because of low power in this group.

(TIFF)

S7 Fig. A) The majority of maternalised alleles (patrepKO and Zfp57KO) are localised away from the periphery with no difference between Gtl2/Meg3 expressing and non-expressing alleles (data presented as a percentage of total alleles). B) Nor were there any significant differences in distribution of expressed and non-expressed alleles when the nucleus was divided into three equal volume bins (an inner, middle and outer; bottom) C) This was similar to WT cells, presented both as C) a proportion of all alleles and D) a proportion of expression state (comparable to Fig 4A in the main text).

(TIFF)
S8 Fig. Double probe DNA FISH in WT ES cells (ES cell line WT8). The images show only one z image plane. All three locations, at which the L1 repeat signal and the Gtl2/Meg2 gene signal have their centres roughly in the same z image plane, are shown as blow-ups on the right. The distance between the signal centres is between 0.25 and 0.36 μm in these three examples.

(TIFF)

S9 Fig. The density of nuclear sizes does not vary considerably between Gtl2/Meg3-expressing and -non-expressing ES cells and therefore does not hint towards a specific down- or upregulation of gene activity during s-phase.

(TIFF)

S1 Table. CSV file of raw data and metadata from all experimental cell lines.

(CSV)

S1 Code. RMarkdown file with code for analysis.

(RMD)

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References

1. van Steensel B, Belmont AS. Lamina-Associated Domains: Links with Chromosome Architecture, Heterochromatin, and Gene Repression. Cell. 2017. https://doi.org/10.1016/j.cell.2017.04.022 PMID: 28525751
2. Cremer T, Cremer C. Chromosome territories, nuclear architecture and gene regulation in mammalian cells. Nature Reviews Genetics. 2001. pp. 292–301. https://doi.org/10.1038/35066075 PMID: 11283701

3. Osborne CS, Chakalova L, Brown KE, Carter D, Horten A, Debrand E, et al. Active genes dynamically colocalize to shared sites of ongoing transcription. Nature Genetics. 2004; 36: 1065–1071. https://doi.org/10.1038/ng1423 PMID: 15361872

4. Nguyen HQ, Bosco G. Gene Positioning Effects on Expression in Eukaryotes. Annual Review of Genetics. 2015;49. https://doi.org/10.1146/annurev-genet-112414-055008 PMID: 26436457

5. Shachar S, Misteli T. Causes and consequences of nuclear gene positioning. Journal of Cell Science. 2017;130. https://doi.org/10.1242/jcs.199786 PMID: 28404786

6. Guelen L, Pagie L, Brasset E, Meuleman W, Faza MB, Talhout W, et al. Domain organization of human chromosomes revealed by mapping of nuclear lamina interactions. Nature. 2008; 453: 948–951. https://doi.org/10.1038/nature06947 PMID: 18463634

7. Kind J, Pagie L, Ortabokoyun H, Boyle S, De Vries SS, Janssen H, et al. Single-cell dynamics of genome-nuclear lamina interactions. Cell. 2013; 153: 178–192 PMID: 23523135

8. Casolari JM, Brown CR, Komili S, West J, Hieronymus H, Silver PA. Genome-wide localization of the nuclear transport machinery couples transcriptional status and nuclear organization. Cell. 2004; 117: 427–439. https://doi.org/10.1016/s0092-8674(04)00448-9 PMID: 15137937

9. Kalverda B, pickinghill H, Shloma V V., Fornerod M. Nucleoporins Directly Stimulate Expression of Developmental and Cell-Cycle Genes Inside the Nucleoplasm. Cell. 2010; 140: 372–383. https://doi.org/10.1016/j.cell.2010.01.011 PMID: 20144760

10. Capelson M, Liang Y, Schulte R, Mair W, Wagner U, Hetzer MW. Chromatin-Bound Nucleolar Components Regulate Gene Expression in Higher Eukaryotes. Cell. 2010; 137: 3067–3077. https://doi.org/10.1242/dev.048231 PMID: 20702563

11. Vaquerizas JM, Suyama R, Kind J, Miura K, Luscombe NM, Akhtar A. Nuclear pore proteins Nup153 and megator define transcriptionally active regions in the Drosophila genome. PLoS Genetics. 2010; 6. https://doi.org/10.1371/journal.pgen.1000846 PMID: 20174442

12. Finlan LE, Sproul D, Thomson I, Boyle S, Kerr E, Perry P, et al. Recruitment to the nuclear periphery can alter expression of genes in human cells. PLoS Genetics. 2008; 4. https://doi.org/10.1371/journal.pgen.1000039 PMID: 18369458

13. Reddy KL, Singh H. Using molecular tethering to analyze the role of nuclear compartmentalization in the regulation of mammalian gene activity. Methods. 2008; 45: 242–251. https://doi.org/10.1016/j.ymeth.2008.06.013 PMID: 18602999

14. Zullo JM, Demarco IA, Piqué-Regí R, Gaffney DJ, Epstein CB, Spooner CJ, et al. DNA sequence-dependent compartmentalization and silencing of chromatin at the nuclear lamina. Cell. 2012; 149: 1474–1487. https://doi.org/10.1016/j.cell.2012.04.035 PMID: 22726435

15. Dialynas G, Speese S, Budnik V, Geyer PK, Wallrath LL. The role of Drosophila Lamin C in muscle function and gene expression. Development. 2010; 137: 3067–3077. https://doi.org/10.1242/dev.048231 PMID: 20702563

16. Kumaran RI, Spector DL. A genetic locus targeted to the nuclear periphery in living cells maintains its transcriptional competence. Journal of Cell Biology. 2008; 180: 51–65. https://doi.org/10.1083/jcb.200706060 PMID: 18195101

17. Reddy KL, Zullo JM, Bertolino E, Singh H. Transcriptional repression mediated by repositioning of genes to the nuclear lamina. Nature. 2008; 452: 243–247. https://doi.org/10.1038/nature06727 PMID: 18272965

18. Ferguson-Smith AC. Genomic imprinting: the emergence of an epigenetic paradigm. Nature Reviews Genetics. 2011; 12: 565–575. https://doi.org/10.1038/nrg3032 PMID: 21765458

19. McEwen KR, Ferguson-Smith AC. Distinguishing epigenetic marks of developmental and imprinting regulation. Epigenetics and Chromatin. 2010; 3. https://doi.org/10.1186/1756-8935-3-2 PMID: 20180964

20. Kota SK, Llères D, Bouschet T, Hirasawa R, Marchand A, Begon-Pescia C, et al. ICR noncoding RNA expression controls imprinting and DNA replication at the Dlk1-Dio3 domain. Developmental Cell. 2014; 31: 19–33. https://doi.org/10.1016/j.devcel.2014.08.009 PMID: 25263792

21. Soares ML, Edwards CA, Dearden FL, Ferron SR, Curran S, Corish J, et al. Targeted deletion of a 170 kb cluster of LINE1 repeats: implications for regional control. Genome research. 2018; gr.221366.117. https://doi.org/10.1101/gr.221366.117 PMID: 29367313
22. Peric-Hupkes D, Meuleman W, Pagie L, Bruggeman SWM, Solovei I, Brugman W, et al. Molecular Maps of the Reorganization of Genome-Nuclear Lamina Interactions during Differentiation. Molecular Cell. 2010; 38: 603–613. https://doi.org/10.1016/j.molcel.2010.03.016 PMID: 20513434

23. Sun B, Ito M, Mendjan S, Ito Y, Brons IGM, Murrell A, et al. Status of genomic imprinting in epigenetically distinct pluripotent stem cells. Stem Cells. 2012; 30: 161–168. https://doi.org/10.1002/stem.793 PMID: 22109880

24. Schoenfelder S, Furlan-Magaril M, Mifsud B, Tavares-Cadete F, Sugar R, Javieire BM, et al. The pluripotent regulatory circuitry connecting promoters to their long-range interacting elements. Genome Research. 2015. https://doi.org/10.1101/gr.185272.114 PMID: 25752748

25. Dixon JR, Selvaraj S, Yue F, Kim A, Li Y, Shen Y, et al. Topological domains in mammalian genomes identified by analysis of chromatin interactions. Nature. 2012; 485: 376–380. https://doi.org/10.1038/nature11082 PMID: 22495300

26. Shi H, Stroganov R, Takahashi N, Kazachenka A, Loricz MC, Hemberger M, et al. ZFP57 regulation of transposable elements and gene expression within and beyond imprinted domains. Epigenetics Chromatin. 2019; 12: 49. https://doi.org/10.1186/s13072-019-0295-4 PMID: 31399135

27. Luo Z, Lin C, Woodfin AR, Bartom ET, Gao X, Smith ER, et al. Regulation of the imprinted Dlk1-Dio3 locus by allele-specific enhancer activity. Genes Dev. 2016; 30: 92–101. https://doi.org/10.1101/gad.270413.115 PMID: 26728555

28. Takada S. Epigenetic analysis of the Dlk1-Gtl2 imprinted domain on mouse chromosome 12: implications for imprinting control from comparison with Igf2-H19. Human Molecular Genetics. 2002; 11: 77–86. https://doi.org/10.1093/hmg/11.1.77 PMID: 11773001

29. Li X, Ito M, Zhou F, Youngson N, Zuo X, Leder P, et al. A Maternal-Zygotic Effect Gene, Zfp57, Maintains Both Maternal and Paternal Imprints. Developmental Cell. 2008; 15: 547–557. https://doi.org/10.1016/j.devcel.2008.08.014 PMID: 18854139

30. Lin SP, Youngson N, Takada S, Seitz H, Paulsen M, et al. Asymmetric regulation of imprinting on the maternal and paternal chromosomes at the Dlk1-Gtl2 imprinted cluster on mouse chromosome 12. Nature Genetics. 2003; 35: 97–102. https://doi.org/10.1038/ng1233 PMID: 12937418

31. Chuang C-H, Carpenter AE, Fuchsova B, Johnson T, de Lanerolle P, Belmont AS. Long-Range Directional Movement of an Interphase Chromosome Site. Current Biology. 2006; 16: 825–831. https://doi.org/10.1016/j.cub.2006.03.059 PMID: 16631592

32. Fraser P, Bickmore W. Nuclear organization of the genome and the potential for gene regulation. Nature. 2007; 447: 413–417. https://doi.org/10.1038/nature05916 PMID: 17522674

33. Llères D, Moindrot B, Pathak R, Piras V, Matelot M, Pignard B, et al. CTCF modulates allele-specific sub-TAD organization and imprinted gene activity at the mouse Dlk1-Dio3 and Igf2-H19 domains. Genome Biol. 2019; 20: 272. https://doi.org/10.1186/s13059-019-1896-8 PMID: 31831055

34. Savatier P, Lapillonne H, Jimanova L, Vitelli L, Samurut J. Analysis of the Cell Cycle in Mouse Embryonic Stem Cells. Embryonic Stem Cells. 2015; 185: 27–33. https://doi.org/10.1385/1-59259-241-4:27 PMID: 270413.115 PMID: 31399135

35. Kind J, Pagie L, De Vries SS, Nahidiazar L, Dey SS, Bienko M, et al. Genome-wide Maps of Nuclear Lamina Interactions in Single Human Cells. Cell. 2015;163. https://doi.org/10.1016/j.cell.2015.03.040 PMID: 26365489

36. Ying Q-L, Ying Q-L, Wray J, Wray J, Nichols J, Nichols J, et al. The ground state of embryonic stem cell self-renewal. Nature. 2008; 453: 519–23. https://doi.org/10.1038/nature06968 PMID: 18497825

37. Giorgetti L, Galupa R, Nora EP, Piolot T, Lam F, Dekker J, et al. Predictive polymer modeling reveals coupled fluctuations in chromosome conformation and transcription. Cell. 2014; 157: 950–963. https://doi.org/10.1016/j.devcel.2008.08.014 PMID: 18854139

38. Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, et al. Fiji: an open-source platform for biological-image analysis. Nature Methods. 2012; 9: 676–682. https://doi.org/10.1038/nmeth.22495300

39. R Core Team. R: A Language and Environment for Statistical Computing. Vienna, Austria: R Foundation for Statistical Computing; 2019. Available: https://www.R-project.org/

40. Bates D, Mächler M, Bolker B, Walker S. Fitting Linear Mixed-Effects Models Using lme4. J Stat Soft. 2015; 67. https://doi.org/10.18637/jss.v067.i01

41. Kuznetsova A, Brockhoff PB, Christensen RHB. Ime4. J Stat Soft. 2017; 82. https://doi.org/10.18637/jss.v082.i13

42. Gelman A, Hill J. Data Analysis Using Regression and Multilevel/Hierarchical Models. Cambridge: Cambridge University Press; 2006. https://doi.org/10.1017/CBO9780511790942

43. Wickham H, Averick M, Bryan J, Chang W, McGowan L, François R, et al. Welcome to the Tidyverse. JOSS. 2019; 4: 1686. https://doi.org/10.21105/joss.01686