The B-type lamin is required for somatic repression of testis-specific gene clusters

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Large clusters of coexpressed tissue-specific genes are abundant on chromosomes of diverse species. The genes coordinately misexpressed in diverse diseases are also found in similar clusters, suggesting that evolutionarily conserved mechanisms regulate expression of large multigenic regions both in normal development and in its pathological disruptions. Studies on individual loci suggest that silent clusters of coregulated genes are embedded in repressed chromatin domains, often localized to the nuclear periphery. To test this model at the genome-wide scale, we studied transcriptional regulation of large testis-specific gene clusters in somatic tissues of *Drosophila*. These gene clusters showed a drastic paucity of known expressed transgene insertions, indicating that they indeed are embedded in repressed chromatin. Bioinformatics analysis suggested the major role for the B-type lamin, LamDm$_o$, in repression of large testis-specific gene clusters, showing that in somatic cells as many as three-quarters of these clusters interact with LamDm$_o$. Ablation of LamDm$_o$ by using mutants and RNAi led to detachment of testis-specific clusters from nuclear envelope and to their selective transcriptional up-regulation in somatic cells, thus providing the first direct evidence for involvement of the B-type lamin in tissue-specific gene repression. Finally, we found that transcriptional activation of the lamina-bound testis-specific gene cluster in male germ line is coupled with its translocation away from the nuclear envelope. Our studies, which directly link nuclear architecture with coordinated regulation of tissue-specific genes, advance understanding of the mechanisms underlying both normal cell differentiation and developmental disorders caused by lesions in the B-type lamins and interacting proteins.

coexpressed genes | nuclear lamina

Previous studies by others and us have shown that many coexpressed tissue-specific genes are organized in large continuous clusters on chromosomes of diverse species (1–3), and similar clustering has been observed for the genes deregulated in diverse diseases (4–8). These findings imply that higher-order chromatin structure may be involved in regulation of extensive multigenic regions in both normal development and its pathological disruptions. In support of this suggestion, alterations in chromatin structure across large multigenic domains have been correlated to changes in gene expression (9–13). Repressed multigenic regions are frequently localized to nuclear periphery (14), and a number of studies linked derepression/activation of genetic loci with their translocation away from the nuclear envelope (15–21). These observations suggest that tethering of genetic loci to nuclear lamina causes their silencing. Recent reports of repression of integrated transgenes and adjacent endogenous genes upon their artificial recruitment to the nuclear envelope (21, 22) support this hypothesis; however, similar studies on different loci did not show repression (23, 24) indicating that silencing at nuclear envelope can be locus-specific. Therefore, although studies on individual loci have suggested that silent tissue-specific gene clusters can be embedded in multigenic chromatin domains repressed at nuclear lamina, it is not clear to what extent this model is applicable to all of the numerous tissue-specific gene clusters present in the genome. In addition, these studies stopped short of elucidating the molecular mechanism(s) that control gene repression within such clusters. In particular, the repressory role of the B-type lamins—the major components of the nuclear lamina (25)—has not been demonstrated.

The possible evolutionarily conserved role of the B-type lamins in developmental gene repression is suggested by interaction of these lamins with silent multigenic regions in both *Drosophila* and mammals (20, 26). This hypothesis is supported by the studies that link mutations in mammalian B-type lamins with developmental disorders such as autosomal dominant leukodystrophy, premature cell senescence, and lipodystrophy (27–29), and by observation of defects suggesting accelerated aging and neuromuscular degeneration in the *Drosophila* mutant for the B-type lamin *LamDm$_0$* (30). Moreover, even a wider spectrum of similar degenerative tissue-specific diseases has been associated with mutations in the nuclear envelope components interacting with both the B- and the A/C-type lamins, and in the A/C-type lamins themselves (31–38). Intriguingly, recent studies strongly imply that such disorders (collectively identified as laminopathies) involve impaired maintenance of stem cells and their aberrant differentiation (39, 40). Thus, accumulating evidence suggests that the nuclear lamina components, and in particular B-type lamins, regulate cell differentiation through repression of numerous multigenic tissue-specific chromatin domains at the nuclear envelope. Our studies presented here support this hypothesis by providing evidence that clusters of testis-specific genes, in general, represent genome regions silenced in somatic cells, and that at least three-quarters of these repressed gene clusters interact with the B-type lamin LamDm$_0$. Further, we directly show that LamDm$_0$ is required for somatic repression of testis-specific gene clusters at nuclear envelope, and that transcriptional activation of such cluster during male germ-line differentiation is coupled with its detachment from the nuclear lamina.

Results and Discussion

Paucity of the Marked Transposon Insertions in the Large Clusters of testes-Specific Genes: Evidence for Somatic Repression of Multigenic Chromatin Domains. Recent studies (14) showed that transcriptionally active multigenic regions support expression of the transgenes integrated therein, whereas transgenes inserted in silent multigenic regions are suppressed. These findings indi-

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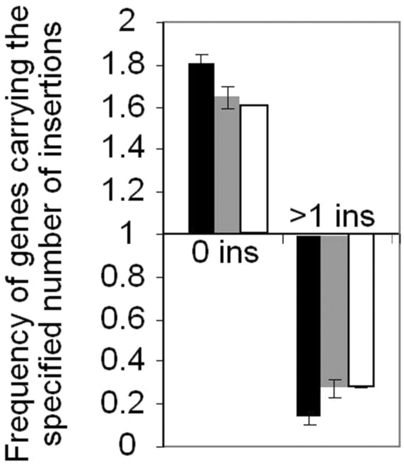

Fig. 1. Paucity of the known marked transposon insertions in large testis-specific gene clusters. The frequency of genes with no known insertions or with multiple known insertions, as indicated on the horizontal axis, is shown. The average frequency of insertions per gene in the entire genome served as the reference. The following gene sets were analyzed: testis-specific genes found in large clusters of 3 or more genes (black bars), in small clusters of 2 genes (gray bars), or not clustered at all (open bars).

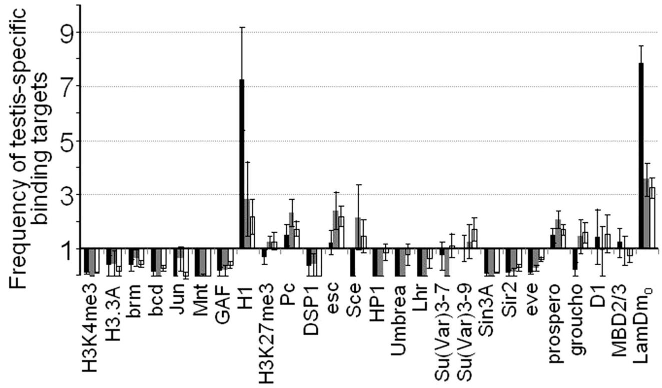

Fig. 2. Selective enrichment of large testis-specific gene clusters with the LamDm$_0$ and histone H1 binding targets. The frequencies of binding targets for the proteins indicated at the bottom were calculated among the testis-specific genes present in large clusters (black bars) or in small clusters (gray bars), or not clustered at all (open bars). The relative increase in binding target frequency is shown compared with the entire genome.

cated that integrated transgenes faithfully report the state of genome regions as permissive or repressive for gene expression. Data from our group and others suggest that clusters of testis-specific genes are embedded in repressed multigenic chromatin domains in somatic tissues (9, 41). If this model is correct for the majority of testis-specific gene clusters in the genome, the transgenes integrated in these clusters should be silenced in somatic cells. To test this prediction, we analyzed the distribution of the 39,894 known transgene insertions identified in saturating mutagenesis screens owing to expression of the transgene-based marker genes in somatic tissues (e.g., 42). Because these insertions mark genome regions permissive for somatic gene expression, the genome regions showing paucity of insertions are likely repressed in somatic cells.

Gene sets for this analysis were generated by using the database of Affymetrix data on gene expression in *Drosophila* tissues (43). Testis-specific genes were identified as showing consistently detectable expression in testes across the 4 replicate microarrays, and no consistent expression in any of the other tissues including adult brain, head, crop, gut, Malpighian tubule, ovaries, male accessory glands, thoracoabdominal ganglia, and thoracic and abdominal carcass, as well as larval Malpighian tubule and salivary gland. Analysis of chromosomal distribution of the testis-specific genes identified 74 large clusters of 3 or more genes (containing a total of 293 genes). In addition, we identified a set of 330 testis-specific genes present in small clusters of 2 genes, and a set of 827 testis-specific genes that are not clustered [supporting information (SI) Table S1, Table S2, and Table S3].

The frequency of transgene insertions in the testis-specific genes was compared with the average frequency across the entire genome. On average, 53% of genes in the *Drosophila* euchromatic genome have been "hit" by at least one transgene insertion, and 37% of genes have been hit more than once. The genes present in large testis-specific clusters did not contain insertions 1.8 times more frequently than the average ($P < 0.0001$, one-tailed z test), and the number of such clustered genes with multiple insertions was more than 7 times lower than the average (Fig. 1, black bars). This paucity of transposon insertions in clustered testis-specific genes is not related to the sizes of analyzed genes, because not only the frequency of insertions per gene, but also the frequency of insertions per kilobase was decreased (>3-fold) within the testis-specific gene clusters, as compared with the entire euchromatic genome. Finally, paucity of known transposon insertions is specifically related to the large gene clusters, because the testis-specific genes that are present in small clusters or not clustered at all showed a significantly lesser reduction in insertion frequency ($P = 0.013$ and $P = 0.002$, respectively, one-tailed z test) (Fig. 1, gray and white bars). These observations strongly suggest that large testis-specific gene clusters are embedded in the repressive genome regions in somatic tissues.

Binding Sites for the B-Type Lamin LamDm$_0$ Are Frequently Associated with Large Testis-Specific Gene Clusters in Somatic Cells. To identify candidate regulators of somatic repression of the testis-specific gene clusters, we analyzed enrichment of testis-specific genes with known binding targets for a number of proteins implicated in transcriptional regulation. The data on protein binding in somatic cells were obtained from the genome-wide surveys (44, 45). In our analysis, we considered genes as binding targets if they showed enrichment in protein binding survey equal to or above the arbitrarily set threshold. The results were similar for 3 different thresholds of 1.5-, 2.0-, and 2.5-fold enrichment (Table S4, Fig. S1); the representative analysis using a threshold of 2.0 is shown on Fig. 2. The average frequencies of binding targets across the euchromatic genome were used as the references.

As expected, general markers of transcriptional activity such as H3K4-me3 and Histone 3.3A and transcriptional activators including Brm, Bcd, Jun, Mnt, and GAF are rarely associated with testis-specific genes in somatic tissues (Fig. 2). In contrast, binding targets of Histone H1, which has been implicated in transcriptional repression (46, 47), are strikingly enriched among the testis-specific genes present in large clusters. Although it remains to be determined whether H1 has an active role in somatic repression of these genes, or merely marks the silent loci as suggested by studies in yeast (48), it is possible that recruitment of this linker histone explains the "closing" of chromatin observed on testis-specific gene clusters in somatic cells (9, 41).

Among the other proteins, large testis-specific gene clusters show binding to the B-type lamin LamDm$_0$ 8 times more frequently than the average ($P < 0.0001$, one-tailed z test). As many as 56% of genes analyzed in these clusters interact with LamDm$_0$, and among the 64 clusters in which at least one gene has been assayed for the LamDm$_0$ binding, three-quarters (47) show such binding with the cutoff of 2.0. Testis-specific genes

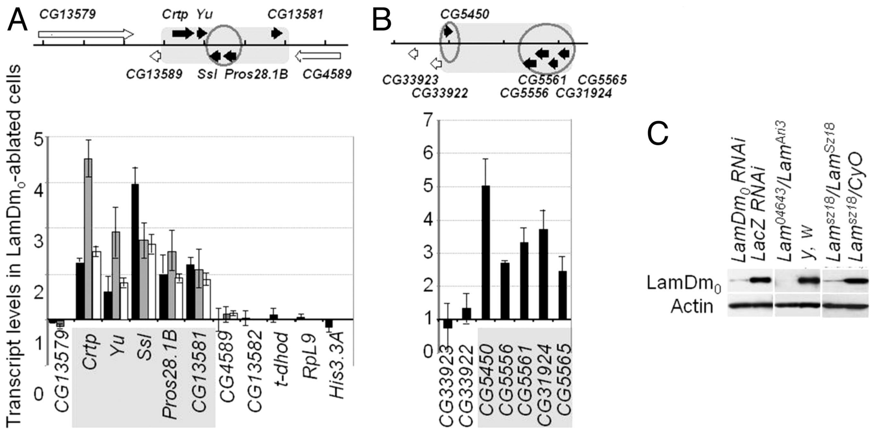

Fig. 3. Ablation of LamDm$_0$ leads to somatic derepression of the testis-specific gene clusters in the *60D1* (*A*) and *22A1* (*B*) regions. (*Upper*) Genome organization of the regions showing testis-specific genes (black) and broadly expressed (white) genes as arrows. Gene clusters are outlined as gray rectangles, and genes showing at least 2-fold enrichment with LamDm$_0$ in somatic cells (20) are outlined by circles. Tick marks correspond to the 5-kb intervals. (*Lower*) Transcript levels in the homozygous *Lam*^{Sz18} first-instar larvae compared with their heterozygous siblings (black bars), in somatic tissues of the *Lam*^{04643}/*Lam*^{ari3} adult females compared with the *y*, *w* flies (gray bars), and in the *LamDm*$_0$ RNAi-treated *S2* cells compared with the *LacZ* RNAi-treated cells (open bars). The *Actin5C* transcript served as a template loading reference. (*C*) Western blot analysis confirms ablation of LamDm$_0$ in RNAi-treated cells, in the *Lam*^{04643}/*Lam*^{ari3} females and in the *Lam*^{Sz18}/*Lam*^{Sz18} larvae.

that are not clustered or are present in small clusters do not show such a drastic enrichment, and both these gene sets show significantly lower incidence of LamDm$_0$-bound genes than large clusters ($P < 0.0001$ for both comparisons, one-tailed *z* test), suggesting that LamDm$_0$ is explicitly involved in somatic repression of extensive multigenic testis-specific domains. Although lamins themselves probably do not regulate gene expression, they probably contribute to gene silencing at nuclear envelope by providing docking sites for diverse repressors (38, 49). Intriguingly, even though heterochromatin protein HP1 has been implicated in gene silencing at nuclear lamina (36, 50, 51), binding targets for HP1 and associated proteins Lhr, Umbrea, Su(Var)3-7, and Su(Var)3-9 (52–55) are not enriched among clustered testis-specific genes. Our analysis also did not show enrichment of this gene set with binding targets of Polycomb (Pc) and Pc group proteins DSP1, Esc, and Sce (56–58), or with the Pc-related trimethylation of H3-K27 (45), despite the previously suggested role for Pc in repression of testis-specific genes (59). Other analyzed repressors (Fig. 2) did not show strongly preferential binding to the clustered testis-specific genes as well. Even though a moderate (2- to 3-fold) enrichment of clustered genes with Groucho, D1, and Su(Var)3-9 binding targets was observed for the low-stringency enrichment cutoff of 1.5, these trends are not consistent at higher cutoff values (Fig. S1, Table S4) and therefore their significance is questionable. Thus, the repressory factors involved in silencing of large testis-specific gene clusters at nuclear envelope remain to be identified. Recent studies showing that silencing of lamina-tethered transgenes is linked to histone deacetylation (21, 22) suggest that gene repression at nuclear envelope role is mediated by HDACs, such as the lamina-associated enzymes HDAC1 and HDAC3 (36, 60). Further studies are required to elucidate the roles of these proteins in tissue-specific repression of lamina-associated multigenic domains.

LamDm$_0$ Is Required for Somatic Repression of the Lamina-Bound Testis-Specific Gene Clusters. Because bioinformatics studies strongly suggested the major role for LamDm$_0$ in somatic repression of the testis-specific gene clusters, we directly tested this hypothesis by analyzing gene expression in somatic cells with ablated LamDm$_0$. We analyzed 2 representative LamDm$_0$-bound testis-specific gene clusters. One cluster of 5 testis-specific genes in cytological region *60D1* was thoroughly characterized in our previous studies, which showed that this cluster is embedded in "closed" chromatin in somatic tissues (9). Another region *22A1* has been identified as a prominent lamina-bound domain in cultured somatic cells (20), and testis-specific expression of 5 genes clustered in this region was confirmed by the real-time RT-PCR (Fig. S2). Expression of the genes included in these 2 clusters was quantitatively studied in the first instar larvae homozygous for the null allele *Lam*^{Sz18} by the real-time RT-PCR. The heterozygous *Lam*^{Sz18}/*CyO,P{Ubi-GFP.S65T}* siblings were identified by their GFP fluorescence and used as the reference. The first instar larvae essentially represented the samples of somatic tissues, because only a few primordial germ cells are present at this early stage of development. We found that the transcript levels for all 5 testis-specific genes in the *60D1* region are significantly higher ($P < 0.01$) in the *Lam*^{Sz18} homozygotes than in their siblings carrying the *CyO,P{Ubi-GFP.S65T}* chromosome which is "wild type" for the *LamDm*$_0$ (Fig. 3*A*, black bars). Similarly, all 5 testis-specific genes in the *22A1* cluster showed strong up-regulation in the *Lam*^{Sz18} mutants (Fig. 3*B*). For comparison, we also analyzed the expression of the testis-specific transcript of the gene *dhod*. The testis-specific *dhod* promoter is juxtaposed to the ubiquitously active promoter of this gene (61), thus the *dhod* gene region is not repressed in somatic tissues. In addition, we analyzed the genes *CG13579*, *CG4589*, *CG13582*, *RpL9*, *His-3.3A*, *CG33922*, and *CG33923*, which are not testis-specific. Neither the testis-specific *dhod* transcript, nor the broadly expressed transcripts of the other analyzed genes were affected in the *Lam*^{Sz18} homozygotes (Fig. 3 *A* and *B*, black bars). These results indicated that loss of LamDm$_0$ leads to selective derepression of testis-specific genes clustered in the *60D1* and *22A1* regions.

We confirmed our observations by analyzing gene expression in the *60D1* region in somatic tissues in which *LamDm*$_0$ was ablated by using a different combination of the alleles, including the null allele *Lam*^{04643} and the hypomorphic allele *Lam*^{ari3}. All testis-specific genes in the *60D1* cluster were significantly ($P < 0.01$) up-regulated in the gonadectomized adult *Lam*^{04643}/*Lam*^{ari3} females, as compared with the gonadectomized *y*, *w* females that are "wild type" for the *LamDm*$_0$. At the same time, the broadly expressed genes *CG13579* and *CG4589* were not affected (Fig. 3*A*, gray bars). The effect of LamDm$_0$ ablation on up-regulation of the testis-specific *60D1* cluster was further corroborated in the RNAi studies on the cultured somatic *S2* cells. In the cells treated with the $LamDm_0$ dsRNA, the testis-specific genes in this cluster showed significant ($P < 0.01$) up-regulation, in contrast to the flanking broadly expressed gene *CG4589* (Fig. 3*A*, open bars). Efficient ablation of $LamDm_0$ in the RNAi-treated cells, as well as in the mutant somatic tissues, was confirmed by Western blot analysis (Fig. 3*C*).

Our observations showed that $LamDm_0$ is required for somatic repression of testis-specific gene clusters. The derepression of testis-specific gene clusters upon ablation of $LamDm_0$ in somatic cells was selective because a number of other genes not included in such clusters were not affected. This effect was different from the global down-regulation of RNA polymerase activity previously reported in the Lamin B1-ablated mammalian cells (62). Such difference is probably due to the high mortality caused by the Lamin B1 ablation (62), whereas in our experiments the reduced levels of $LamDm_0$ were physiologically acceptable as evidenced by the survival of the Lam^{04643}/Lam^{ari3} mutants through adulthood. Thus, our studies, which took advantage of *Drosophila* that can tolerate severe changes in the B-type lamin dosage, provided unique direct experimental evidence that this major component of nuclear lamina is involved in repression of tissue-specific multigenic domains.

Transcriptional Up-Regulation of the Lamina-Bound Testis-Specific Clusters Is Coupled with Their Detachment from the Nuclear Envelope. We hypothesized that, in addition to the somatic silencing of testis-specific gene clusters, $LamDm_0$ is also required for attachment of these clusters to the nuclear envelope. To test this hypothesis, we determined intranuclear positions of the *60D1* and *22A1* regions in the interphase nuclei of cultured *S2* cells in which $LamDm_0$ was ablated by RNAi. Fluorescence in situ hybridization (FISH) combined with immunostaining for $LamDm_0$ confirmed RNAi-induced ablation of $LamDm_0$, and showed approximately 2-fold decrease in frequency of the *60D1* and *22A1* loci bound to lamina (Fig. 4 *A* and *B* and Fig. S3). Next, we analyzed association of the *60D1* gene cluster with nuclear envelope during male germ-line differentiation. The whole-mount testes dissected from the third instar larvae were analyzed for intranuclear localization of the *60D1* region by FISH combined with immunostaining for $LamDm_0$. Morphologically, a group of small cells is located at the end of testis (lower left on Fig. 4*C*) and includes spermatogonia, cyst cells and stem cells, in which the *60D1* locus is silent (9). In these cells, the *60D1* region is associated with nuclear lamina in 76% of nuclei (Fig. 4*D*), similarly to the cultured somatic *S2* cells (Fig. 4*B*). On the contrary, in spermatocytes (identified by characteristic large nuclei, Fig. 4*C*, upper right corner) in which testis-specific genes in the *60D1* region are expressed (9), this region is found at the nuclear envelope in only 6% of the nuclei (Fig. 4*D*). Thus, transcriptional activation of the testis-specific gene cluster in male germ line is coupled to its dissociation from the nuclear envelope. Similarly, detachment from the nuclear lamina has been associated with transcriptional activation of other lamina-bound loci both in *Drosophila* and in mammals (20, 21). These observations strongly suggest a model in which gene repression is controlled in a cell type-specific manner through regulated tethering of chromatin to the nuclear lamina. Further dissection of the mechanisms that mediate repression of lamina-bound multigenic regions and control localization of these regions at nuclear envelope will provide new insights into coordinated regulation of tissue-specific genes, thus advancing understanding of cell differentiation both in normal development and in disease.

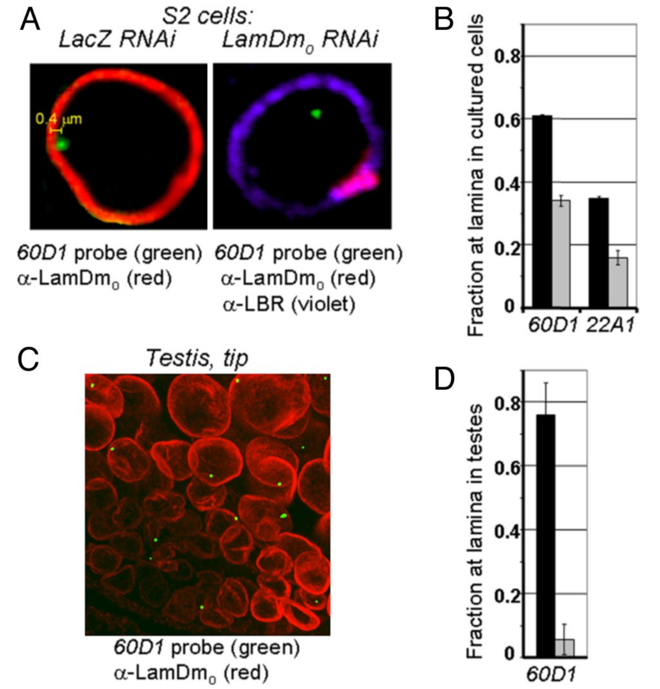

Fig. 4. Expression of the $LamDm_0$-bound testis-specific loci is coupled to their detachment from the nuclear envelope. (*A*) FISH shows localization of the testis-specific *60D1* locus within 0.4 μm from the nuclear envelope revealed by immunostaining for $LamDm_0$, indicating direct contact between the locus and the envelope in cultured *S2* cells treated with the control *LacZ* dsRNA. In the cells treated with the $LamDm_0$ dsRNA, $LamDm_0$ is ablated and the *60D1* locus is detached from the nuclear envelope (revealed by immunostaining for dLBR). (*B*) Quantitative analysis shows that $LamDm_0$ dsRNA treatment decreases the frequency with which the testis-specific *60D1* and *22A1* loci are found in contact with nuclear envelope, as compared with the control *LacZ* dsRNA treatment (gray versus black bars). (*C* and *D*) The *60D1* locus is found in contact with nuclear envelope in the majority of small cells including spermatogonia, cyst cells, and stem cells located at the testis tip (*C*, lower left; *D*, black bar) but not in the larger spermatocytes (*C*, upper right; *D*, gray bar).

Methods

Detailed descriptions of procedures are included in *SI Methods* and Table S5.

***Drosophila melanogaster* Stocks.** *y, w*; Lam^{sz18} (63) and Lam^{04643} (64) mutants and the stock containing the *CyO* balancer chromosome carrying the *P{Ubi-GFP.S65T}* expression transgene were obtained from the Bloomington *Drosophila* Stock Center at Indiana University, and the Lam^{Ari3} mutant (65) was kindly provided by J. Fischer (University of Texas, Austin).

Cell Culture and RNAi. *Schneider-2* (*S2*) cells were grown in Schneider's *Drosophila* medium supplemented with 10% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin at 26 °C. To obtain dsRNAs, coding sequences of *Escherichia coli lacZ* gene (≈650 bp) and *D. melanogaster* $LamDm_0$ gene (≈800 bp) were amplified from the pCaSpeR-βgal (67) and J14–3A (63) plasmids, and transcribed in vitro by using T7 RNA polymerase (Promega). Treatment of cells with dsRNA was performed by using a previously described protocol (66).

Reverse Transcription and Quantitative PCR. Total RNA was obtained by extraction with the TRIzol reagent (Invitrogen), and contaminating DNA was removed by DNase I treatment. Real-time RT-PCR assays were performed on cDNA synthesized with the oligo(dT) primer, using SYBR Green chemistry (Applied Biosystems) and the Mx3000P hardware (Stratagene). Acrylamide gel-based assay used cDNA synthesized with the random hexamer primer; reactions were run in the presence of ^{33}P-dATP and reaction products were quantified with PhosphorImager Storm-820 (Molecular Dynamics).

Western Blot Analysis. Proteins were extracted with 8 M urea, 1 M Tris·HCl, pH 7.0. $LamDm_0$ was detected with the monoclonal murine antibody ADL67 (68), and β-actin, with the polyclonal murine antibody (Abcam).

Immuno-FISH. FISH probe for the *60D1* region was prepared by using the ≈35 kb cosmid k9 (9). The probe for the *22A1* region was generated by PCR amplification of 4 tiling ≈5-kb fragments representing a ≈20 kb genome segment. Digoxigenin-labeled DNA probes were hybridized with cells or whole-mount larval testes as described in ref. 69 with some modifications, and immunostained by using rabbit polyclonal antibody against $LamDm_0$ (no. 836) (70) or guinea pig polyclonal anti-dLBR antibody (71), and anti-DIG-FITC-conjugated antibody (Roche). Secondary Alexa 546- or Alexa 647-conjugated antibodies were from Invitrogen.

Image Analysis. Three-dimensional image stacks were recorded with a confocal Zeiss LSM510 Meta microscope. Images were processed and analyzed by using IMARIS 5.0.1 software (Bitplane AG) to measure the distance between the FISH signal and the reconstructed nuclear surface. The distance of 0.4 μm or less was interpreted as localization of FISH signal at nuclear envelope, because both the nuclear lamina and the FISH signal had a visual width of $\approx$0.4 μm.

Analysis of Transposon Insertions and Protein Binding. Gene spans included transcribed regions and, in addition, the 500-bp 5′-UTR segments to account for the proximal promoter regions. Coordinates of the gene spans, and of the 39,894 known transgenes insertions were obtained from the *Drosophila* genome database (www.flybase.net). Genes were analyzed for the number of insertions within the gene span, using custom-made software. Raw gene microarray data on binding of diverse chromatin proteins were obtained from the survey of de Wit *et al.* (44) and publications referenced therein.

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