RESEARCH ARTICLE

Lamin C and chromatin organization in *Drosophila*

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Abstract

Drosophila lamin C (LamC) is a developmentally regulated component of the nuclear lamina. The *lamC* gene is situated in the fifth intron of the essential gene *tout velu* (*ttv*). We carried out genetic analysis of *lamC* during development. Phenotypic analyses of RNAi-mediated downregulation of *lamC* expression as well as targeted misexpression of lamin C suggest a role for *lamC* in cell survival. Of particular interest in the context of laminopathies is the caspase-dependent apoptosis induced by the overexpression of lamin C. Interestingly, misexpression of lamin C in the central nervous system, where it is not normally expressed, did not affect organization of the nuclear lamina. *lamC* mutant alleles suppressed position effect variegation normally displayed at near-centromeric and telomeric regions. Further, both downregulation and misexpression of lamin C affected the distribution of heterochromatin protein 1. Our results suggest that *Drosophila lamC* has a tissue-specific role during development and is required for chromatin organization.

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Introduction

Lamins are the components of a filamentous network underlying the inner nuclear membrane termed the nuclear lamina, and are also located in the interior of the nucleus. The lamina is an important determinant of interphase nuclear architecture as it maintains the integrity of the nuclear envelope and provides anchoring sites for chromatin. Lamins have essential functions in the organization of DNA replication, transcription and RNA splicing, as well as in apoptosis (reviewed in Cohen *et al*. 2001; Goldman *et al*. 2002; Dechat *et al*. 2008; Parnaik 2008). Two major kinds of lamins are present in vertebrate cells: B-type lamins (B1 and B2), found in nearly all somatic cells; and A-type lamins (A and C), expressed in differentiated cells of several lineages. Lamins belong to the intermediate filament family of proteins and have a short N-terminal head domain followed by a α-helical rod and a globular tail (Herrmann *et al*. 2007). Lamin genes are highly conserved across species. *Drosophila melanogaster* has two lamin genes, the B-type homologue *lamin Dm$_0$ (lamDm$_0$)*, expressed in all cell types (Gruenbaum *et al*. 1988); and *lamin C (lamC)*, that exhibits tissue-specific expression like the vertebrate A-type lamins (Bossie and Sanders 1993; Riemer *et al*. 1995; Melcer *et al*. 2007).

Mutations in human *lamin A* gene (*LMNA*) cause at least nine debilitating diseases that affect specific tissues and have been collectively termed laminopathies (reviewed by Worman and Courvalin 2005; Broers *et al*. 2006; Capell and Collins 2006). Most mutations affect skeletal and cardiac muscles, causing autosomal dominant Emery–Dreifuss muscular dystrophy (EDMD), dilated cardiomyopathy and limb-girdle muscular dystrophy. Other mutations cause loss of white fat, bone disorders and peripheral neuropathy. Majority of mutations are missense mutations; those affecting muscle tissue occur throughout the gene, whereas mutations causing abnormalities in fat tissue tend to be clustered towards the carboxy terminus. Valuable insights into lamin A function have been obtained by the knock-down of mouse *LMNA* (Sullivan *et al*. 1999). Mice that lack *LMNA* develop severe muscle wasting, similar to human EDMD and loss of white fat, by 3–4 weeks, and die by eight weeks after birth. It has been suggested that abnormal nuclear struc-

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tures might make cells more susceptible to physical stress (Sullivan *et al*. 1999; Lammerding *et al*. 2004), lead to defects in chromatin organization and altered gene expression (Wilson 2000; Nikolova *et al*. 2004; Capanni *et al*. 2005) and affect signalling pathways during myoblast differentiation (Muralikrishna *et al*. 2001; Mariappan and Parnaik 2005; Parnaik and Manju 2006). Consistently, several studies suggest that interactions between lamins and their binding partners might influence chromatin structure and gene activity (reviewed by Goldman *et al*. 2002; Zastrow *et al*. 2004). Other *Drosophila* nuclear membrane proteins such as young arrest (YA) and otefin interact with lamin Dm_0 and are likely to be involved in nuclear envelope assembly (Goldberg *et al*. 1998).

Drosophila lamC is situated in the fifth intron of another gene, *tout velu* (*ttv*), which codes for a protein involved in the synthesis of heparan sulphate proteoglycan (Bellaiche *et al*. 1998; The *et al*. 1999). Heat-induced overexpression of a *Drosophila* lamin C cDNA construct with a modified C-terminus resulted in melanotic tumours and larval death (Stuurman *et al*. 1999). Mutations in *lamC* that are lethal at the pre-pupal stage have been reported (Schulze *et al*. 2005), but a detailed analysis of *lamC* functions is not yet available.

Results reported here suggest that misregulation of *lamC* affects nuclear organization in specific tissues during development, leading to apoptotic cell death. Further, *lamC* mutant alleles suppress position effect variegation (PEV) and both downregulation and overexpression of *lamC* alter the localization of heterochromatin protein 1 (HP1), thus supporting a role for lamin C in chromatin organization.

Materials and methods

Drosophila stocks and genetic analysis

Recombinant chromosomes and combinations of GAL4 drivers and UAS lines, different mutations and markers were generated using standard genetic techniques. Several flystocks were obtained from various sources: *lamC::GFP PTT-G00158* (Morin *et al*. 2001), ttv^{11904} ttv^{00681}, ttv^{14030}, *Df(2R)trix, hs-FLP, noc/CyO, ey*-FLP, P(FRT)42D and *GMR*-RFP were obtained from Bloomington Stock Center, Indiana, USA; $lamC^{EX296}$ and $lamC^{EX187}$ were kindly provided by L. L. Wallrath (Schulze *et al*. 2005); *sh631* line was from S. X. Hou (Oh *et al*. 2003) and *NP3088* was obtained from the *Drosophila* Genetic Resource Center, Kyoto, Japan. GAL4 strains used are *ap*-GAL4 and *pnr*-GAL4 (Calleja *et al*. 1996), *hs*-GAL4 (personal communication to FlyBase, 2003.5.27), *omb*-GAL4 (personal communication to FlyBase, *Calleja*, 1996.10.16), *ptc*-GAL4 (Brand and Perrimon 1993), *sd*-GAL4 (Shyamala and Chopra 1999) and *Ubx*-GAL4 (Pallavi and Shashidhara 2003). UAS-GFP is reported in Halfon *et al*. (2002). *elav*-GAL4 and *sca*-GAL4 are from Bloomington Stock Center, Indiana USA.

UAS-lamC and $lamC^{RNAi}$ transgenes

A full-length lamin C cDNA construct (LD 31805; Berkeley *Drosophila* Genome Project) was subcloned into pUAST vector (Brand and Perrimon 1993). The construct was first sequenced to ensure that no mutations have been introduced during cloning. Transgenic lines were generated using standard methods. Two independent transgenic flies were obtained. The unique combination of pUAST primer and outward primer from the cloned cDNA allowed amplification of lamin C cDNA only from the transgene and their sequence was further verified. Both the insertions showed similar effects and Western blot analysis showed substantial increase in lamin C levels only when induced with *hs*-GAL4.

The unique RNAi target sequences specific for 3′UTR and tail region of *lamC* were PCR-amplified from cDNA templates using specific primers with incorporated restriction sites (shown below in small letters). The primers used were as follows:

lamin C-UTR

forward 5′ ggaattcAGGATGTTGCCAGCTACGAC 3′

reverse 5′ gaatgcGGCCGCCCAAAATGCATGTTC 3′

lamin C-tail

forward 5′cggaattcCGCATTCGGGAGCTGGAGAACCTC3′

reverse 5′ GGTGTGGCTGGAAACGTTG 3′

The lamin C-UTR (1840–2311) and lamin C-tail (1121–1889) segments of *lamC* were subcloned initially into pMOS vector. Sequences were verified and then subcloned into pSympUAST vector (Giordano *et al*. 2002). Transgenic lines were generated using standard methods. Out of 15 independent transgenic flies generated and examined, two transgenic lines were used for all the studies based on the consistency in generating phenotypes.

Immunohistochemistry

Rabbit polyclonal antibodies to lamin C and guinea pig polyclonal antibodies to lamin Dm_0 were raised to the bacterially expressed *Drosophila* proteins. Monoclonal antibodies to lamin C (LC 28.26; Riemer *et al*. 1995) and to HP1 (C1A9; James *et al*. 1989) were obtained from the Developmental Studies Hybridoma Bank (University of Iowa, Iowa, USA). Their specificities were confirmed by Western blot analysis and immunofluorescence assays. Furthermore, in the CNS, where lamin C is not expressed, we observed staining of the nuclear envelope only with anti-lamin Dm_0 antibodies (see below). Monoclonal antibodies to β-galactosidase were from Sigma (St Louis, USA). Rabbit polyclonal antibodies against *Drosophila* GAGA factor was from R. Mishra (CCMB, Hyderabad, India). Immunochemical staining on imaginal discs and on polytene chromosomes was performed as described earlier (Patel *et al*. 1989; Lavrov *et al*. 2004). For double antibody staining, a combination of (i) monoclonal anti-lamin C and polyclonal anti-lamin Dm_0, (ii) polyclonal anti-lamin C and monoclonal anti-HP1 and (iii) polyclonal anti-lamin C and monoclonal anti-β-galactosidase antibodies were used.

Fluorescence images were obtained either on a Zeiss Apotome^{TM} microscope or Zeiss LSM/Meta Confocal microscope (Jena, Germany). The control and experimental images were digitized at identical microscope and camera settings. The quantifications were done using Image J software (NIH, Washington, USA). The adult appendages were processed for microscopy as described earlier (Shashidhara *et al*. 1999).

Position-effect-variegation assay

For assaying dominant suppression of PEV, an inversion stock *In(1)w^{m4h}; Su(var)205^{5}/In(2L)Cy, In(2R)Cy, Cy^{1}* (Bloomington Stock Center #6234) and telomeric PEV insertion *39C-72* (kindly provided by L. L. Wallrath, University of Iowa, Iowa, USA) were used. As a third PEV marker, variegating *Sb* locus with a pericentromeric heterochromatin insertion *T(2;3)Sb^{V}* (Sinclair *et al*. 1983) was used. The *lamC* excision females, of genotypes as shown in Results section, were crossed with males of PEV lines. The progeny were assayed for suppression of PEV as described (Reuter *et al*. 1982; Cryderman *et al*. 1999). Progeny of *w^{1118}* females crossed to males of PEV lines were used as control for comparison.

Results

The genomic locus of *Drosophila lamC* is within the fifth intron of the essential gene *ttv* in the opposite orientation, at position 51B1, on the right arm of chromosome 2 (see figure 1A). Several lethal P-element insertions (*ttv^{11904}, G00158, NP3088* and *sh631*) have been mapped to the 5′ region of *lamC* gene (shown in figure 1B). However, complementation tests showed that all of them were lethal with *ttv*-specific mutant allele *ttv^{00681}*. Schulze *et al*. (2005) have reported the isolation of two excision alleles of *lamC, lamC^{EX187}* and *lamC^{EX296}*, both of which are viable over *ttv^{00681}* and show near complete reduction in the levels of lamin C protein. They both were reported to be prepupal lethal when brought in trans over the deficiency *Df(2R)trix*. We have observed that both *lamC^{EX187}* and *lam^{CEX296}* alleles are early larval lethal when homozygous and are late larval or early pupal lethal only when hemizygous with *Df(2R)trix*. Considering that these alleles could be rescued by UAS-lamin C transgene (Schulze *et al*. 2005), these discrepancies indicate complexities of the *lamC* locus. We, therefore, employed RNAi-mediated gene knock-down approach to examine the effect of downregulation of *lamC* in more detail.

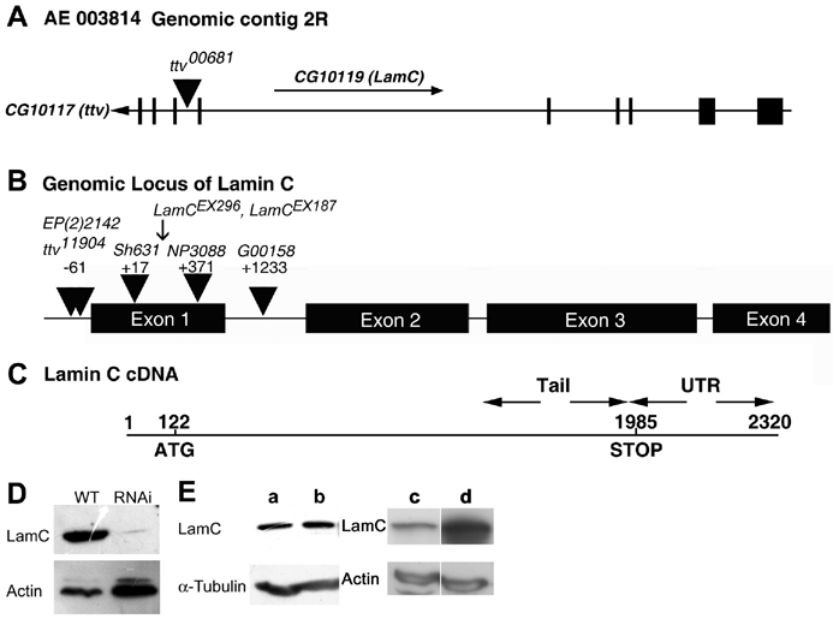


Figure 1. Schematic representation of the *lamC* locus. (A) The genomic contig harbouring the *lamC* and *ttv* genes is indicated (not to scale). (B) The *lamC* genomic locus. The exact insertion sites of various P-elements used in this study have been derived by PCR mapping and are numbered with respect to the *lamC* transcription-initiation site, which was determined by primer extension analysis (data not shown). (C) The positions of the fragments used in RNAi experiments are marked by arrows in the lamin C cDNA. (D) Western blot analysis, for lamin C levels in wild-type (WT) and in *hs*-GAL4/UAS-*lamC*-UTR^{RNAi}; UAS-*lamC*-tail^{RNAi} (RNAi) larvae continuously grown at 29°C. (E) Western blot analyses for lamin C levels in wild-type larvae grown at 29°C (a) and *hs*-GAL4/UAS-lamin C larvae grown at 25°C (b) In another Western blot analysis, lamin C levels were estimated in wild-type (c) and *hs*-GAL4/UAS-lamin C (d) larvae both grown at 29°C and given a pulse of heat-shock at 37°C for 30 min (e) Actin and α-tubulin were used as control for normalizing loading differences.

Defects associated with RNAi-mediated downregulation of lamin C

Two RNAi target regions that do not have significant homology of more than 18 bp with any other *Drosophila* gene (including *lamDm*$_0$) were chosen for generating UAS-*lamC*^{RNAi} transgenes. Regions corresponding to 3′UTR and tail sequences were used to generate RNAi constructs (figure 1C). *hs*-GAL4-driven expression of one copy of either UAS-*lamC-UTR*^{RNAi} or UAS-*lamC-tail*^{RNAi} construct did not affect viability or induce any phenotypes, when expressed in wild-type background. We examined the effect of downregulation of *lamC* function using both the RNAi transgenes together. Constitutive expression of both *lamC-UTR*^{RNAi} and *lamC-tail*^{RNAi} together using *hs*-GAL4 driver resulted early larval lethality. The Western blot analysis using the early larvae showed significant reduction in the levels of lamin C protein (figure 1D).

Combined expression of both the RNAi constructs in the dorsal compartment of the wing disc using *ap*-GAL4 driver resulted in significant loss of lamin C (figure 2C). These discs also showed depletion in lamin Dm$_0$ (figure 2C), suggesting that loss of lamin C caused disruption of the nuclear lamina. This was associated with lethality (upto 25%; n = 161) and adult phenotypes (in 12% flies), which were typically, loss of thoracic bristles (figure 2E) and necrotic patches on the wing blade (figure 2H). Similar phenotypes were observed when UAS-*lamC-UTR*^{RNAi} or UAS-*lamC-tail*^{RNAi} alone was expressed in *lamC*^{EX296} heterozygous backgrounds (figure 2F). Combined expression of both the RNAi transgenes (figure 2I) or one copy of the RNAi transgene in *lamC*^{EX296} background using *ptc*-GAL4 driver resulted in mild curled-wing phenotype (data not shown). These effects can be attributed to cell death resulting from downregulation of lamin C expression.

Effects of tissue-specific overexpression of lamin C

We examined the phenotypes caused by the overexpression of lamin C under a wide range of tissue-specific enhancers using the UAS/GAL4 system (figure 1E).

We first examined if overexpression of lamin C in tissues, where it is normally not expressed, affects nuclear lamina organization or tissue development. Interestingly, *elav*-GAL4 mediated expression of lamin C in the CNS, where it is normally not expressed (figure 3A), led to incorporation of lamin C into the nuclear periphery in a normal rim pattern (figure 3B), but did not affect CNS development and adult flies were normal. To check if lamin C overexpression affects the organization of the nuclear lamina in the wing disc, we overexpressed lamin C using *Ubx*-GAL4 driver, which is expressed in the peripodial cells of the wing disc (figure 3C). We then examined the effect of overexpression of LamC on tissue development by expressing it in developing wing using *omb*-GAL4 and *ap*-GAL4 drivers. We observed distinct phenotypes depending on the tissue and domain of overexpression of lamin C, such as ectopic veins, thickening of veins and loss of the intervein tissue or defective thoracic closure (data not shown). Considering the overexpression of LamC induces wing phenotypes, the absence of phenotype when LamC is overexpressed in the developing CNS reflects its tissue-specific role.

We then examined the effect of overexpression of LamC on the organization of the nuclear lamina. Peripodial cells are squamous epithelial cells, which are large compared to other columnar epithelial cells and thereby it would be easier to examine the effect of lamin C overexpression on the nuclear lamina. Normally, epithelial cells show smooth peripheral staining of the lamins in the nuclei (figure 3,D&F). *Ubx*-GAL4 mediated lamin C expression in the wing disc gave rise to aberrant nuclear morphology with distinct aggregates of lamin C and lamin Dm$_0$ at the nuclear periphery (figure 3,E&G).

We then examined the effect of overexpression of lamin C in developing muscles in view of the muscle-specific defects observed with lamin A/C mutations in humans and mice. Lamin C expression mediated by the early muscle-specific GAL4 driver, 24B-GAL4, led to severe defects in the formation of larval longitudinal muscles (figure 3I) and, probably as a consequence, lethality at the first instar larval stage. *pnr*-GAL4 mediated lamin C expression led to disruption in organization of dorsal indirect flight muscles in the adult (figure 3K).

The above described effects of lamin C overexpression in epithelial and muscle cells suggest cellular lethality (consistent with aberrant nuclear morphology). We, therefore, examined if lamin C overexpression causes cell death, in particular apoptosis. For this purpose, we expressed lamin C in a small domain of the wing disc using *ptc*-GAL4 driver, which expresses only in the A/P boundary cells (figure 4A). Staining of these wing discs with acridine orange suggested extensive apoptotic cell death along the A/P boundary, where lamin C is overexpressed (figure 4C), probably as a consequence the A/P boundary itself was much narrower compared to wild-type discs (figure 4D). We observed highly condensed nuclei, and there was a distinct change in the normal localization of both lamin C and lamin Dm$_0$ (figure 4E; shown at higher magnification in figure 4F). These effects were restricted to the *ptc*-GAL4 domain of expression and the surrounding cells displayed normal staining of lamins C and Dm$_0$. The apoptosis caused by the overexpression of lamin C was further confirmed as the bacculovirus caspase inhibitor, P35, was able to inhibit the cell death and restore normal nuclear morphology (figure 4G). The width of the A/P boundary was also comparable to the wild-type (figure 4G).

Alterations in chromatin structure due to overexpression of lamin C

Several studies suggest that interactions among lamins and their binding partners might influence chromatin structure

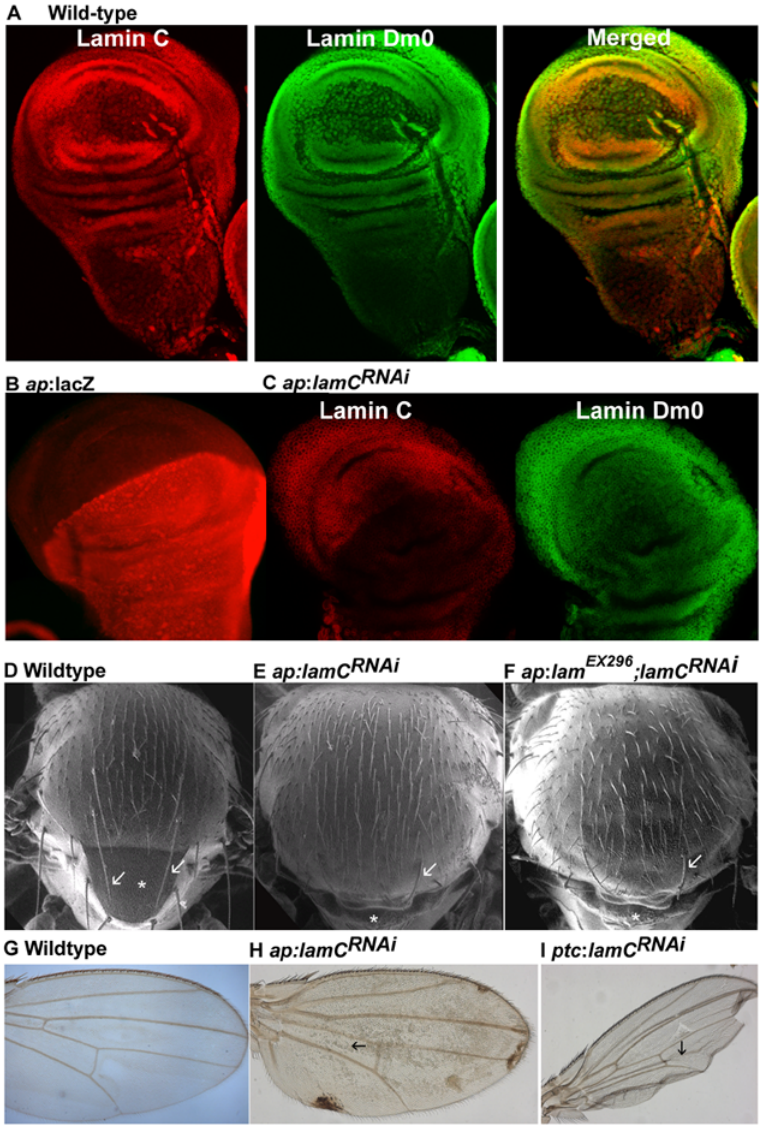


Figure 2. Larval and adult phenotypes caused by RNAi-mediated down regulation of lamin C expression. (A) Wild-type wing disc stained with monoclonal antibodies to lamin C and polyclonal antibodies to lamin Dm_0. (B) ap-GAL4/UAS-GFP wing discs showing the expression pattern of *ap*-GAL4 in wing discs. It is expressed only in dorsal cells of the disc. (C) *ap*-GAL4/UAS-*lamC-UTR*^{RNAi}; UAS-*lamC-tail*^{RNAi} wing disc stained with antibodies to lamin C (red) and lamin Dm_0 (green). Note levels of lamin C are considerably down regulated. There is a decrease in lamin Dm_0 levels (also see figure 8). (D) Adult thorax of wild-type fly. (E) *ap*-GAL4/UAS-*lamC-UTR*^{RNAi}; UAS-*lamC-tail*^{RNAi} fly. Note, loss of scutellum (asterix) and shortening of thoracic macrochaete (sensory organs; arrows). (F) Adult thorax of ap-GAL4/*lamC*^{EX296}; UAS-*lamC-UTR*^{RNAi} fly with similar thoracic phenotypes. Similar phenotypes were observed in *ap*-GAL4/*lamC*^{EX09}; UAS-*lamC-UTR*^{RNAi} flies. One copy of UAS-*lamC-UTR*^{RNAi} on its own does not have any phenotype. (G-I) Adult wing blades of wildtype (G), *ap*-GAL4/UAS-*lamC-UTR*^{RNAi}; UAS-*lamC-tail*^{RNAi} (H) and *ptc*-GAL4/UAS-*lamC-UTR*^{RNAi}; UAS-*lamC-tail*^{RNAi} (I) flies. Down regulation of lamin C causes cell death (arrow in H) and aberrant vein patterns (arrow in I) and also affects wing size (I).

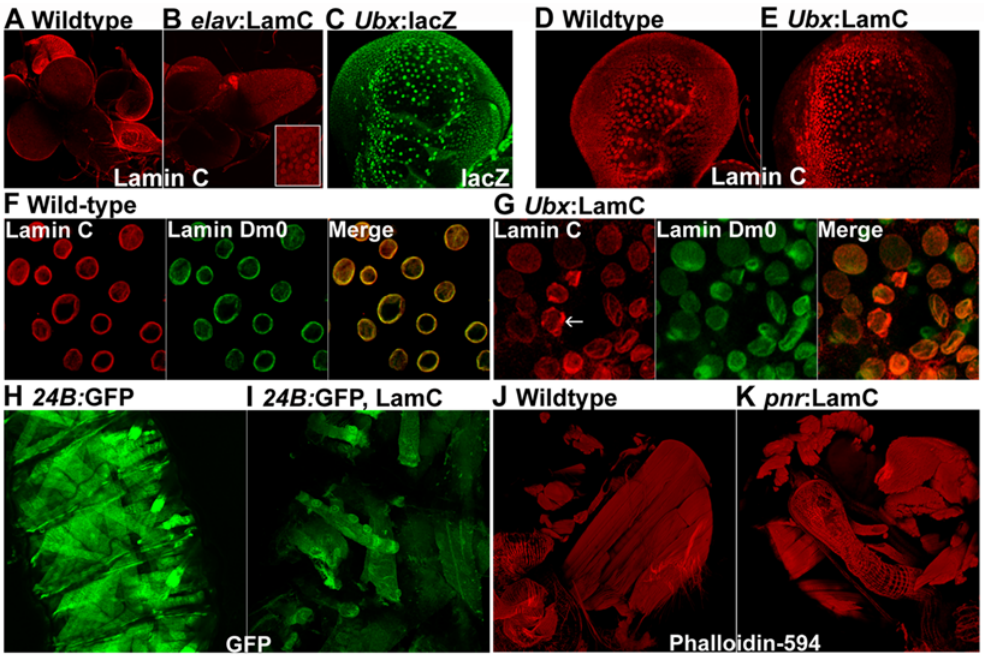


Figure 3. Larval and adult phenotypes caused by the misexpression of lamin C under various tissue-specific enhancers. (A, B) Wild-type (A) and *elav*-GAL4/UAS-lamin C (B) third instar larval CNS. Normally, lamin C is not expressed in the larval CNS and its misexpression is not associated with any phenotype. (C) *Ubx*-GAL4/UAS-GFP wing disc showing GAL4 expression in the peripodial cells. (D, E) Wild-type (D) and *Ubx*-GAL4/UAS-lamin C (E) wing discs stained with antibodies to lamin C. (F, G) Wild-type (F) and *Ubx*-GAL4/UAS-lamin C (G) peripodial cells shown at higher magnification. The cells are stained with antibodies to lamin C (red) and lamin Dm_0 (green); arrow in G points to aggregates of lamin C. (H, I) Larval body wall muscles of wild-type (H) and *24B*-GAL4/lamin C (I) third instar larvae. Muscles in both the images are visualized with the help of UAS-GFP. Note, severe developmental defects in both longitudinal and transverse muscles when lamin C is overexpressed. (J, K) Adult thoracic muscles of wild-type (J) and *pnr*-GAL4/UAS-lamin C (K) flies stained with phalloidin-594. Note, all indirect flight muscles are completely absent when lamin C is overexpressed.

and gene activity (reviewed by Goldman *et al*. 2002; Zastrow *et al*. 2004). However, there is no *in vivo* evidence for the role of lamin C in chromatin organization.

In *Drosophila*, changes in chromatin organization and structure can easily be studied by monitoring the phenomenon of PEV. Inversion-mediated relocalization or transgenic-mediated insertion of *white*$^+$, a euchromatin gene, near centromeric or telomeric heterochromatin results in PEV, which can be demonstrated by the mosaic or variegated expression of the red eye colour (figure 5,A&B). PEV has been attributed to *cis*-spreading of the heterochromatic state and is influenced by chromatin structure and nuclear organization (reviewed by Wallrath 1998). Genes associated with PEV have been identified by analysis of suppression of PEV (reviewed by Weiler and Wakimoto 1995). To determine whether mutations in *lamC* have a direct effect on PEV, $lamC^{EX296}$ and $lamC^{EX187}$ excision alleles (both the alleles are in homozygous w^{1118} background) were crossed to strains bearing transgenes of the *white*$^+$ marker at near-centromeric (*In(1)*w^{m4h}; *Su(var)*205^5*/In(2L)Cy, In(2R)Cy,* Cy^1; here referred to as *In(1)*w^{m4h}) and telomeric (*39C-72*, fourth chromosome) locations, and the progeny were scored for the modification of PEV. Nearly 50% of the progeny showed increased pigmentation (figure 5,A&B). w^{m4h}; *Su(var)205/*$lamC^{EX187}$ had nearly normal red eye colour with little evidence of variegation, in contrast to the variegated eyes of the PEV strains crossed to w^{1118} strains (figure 5,A&B). Precise excisions of ttv^{11904} from which $lamC^{EX296}$ and $lamC^{EX187}$ were generated, did not show any suppression of PEV associated with *In(1)*w^{m4h} and telomeric *39C-72* (data not shown), suggesting specificity of the effect to *lamC* mutants. Similar suppression of PEV at the *Sb* locus with a pericentromeric heterochromatin insertion (*T(2;3)*Sb^V; Sinclair *et al*. 1983 was observed by $lamC^{EX296}$ and $lamC^{EX187}$ (figure 5C). The suppression of PEV by *lamC* mutants strongly suggests that normal lamina organization is required for PEV.

HP1 is mislocalized in loss and gain of lamin C backgrounds

HP1 is a non-histone-chromosomal protein enriched in heterochromatin that is involved in the transcriptional silencing

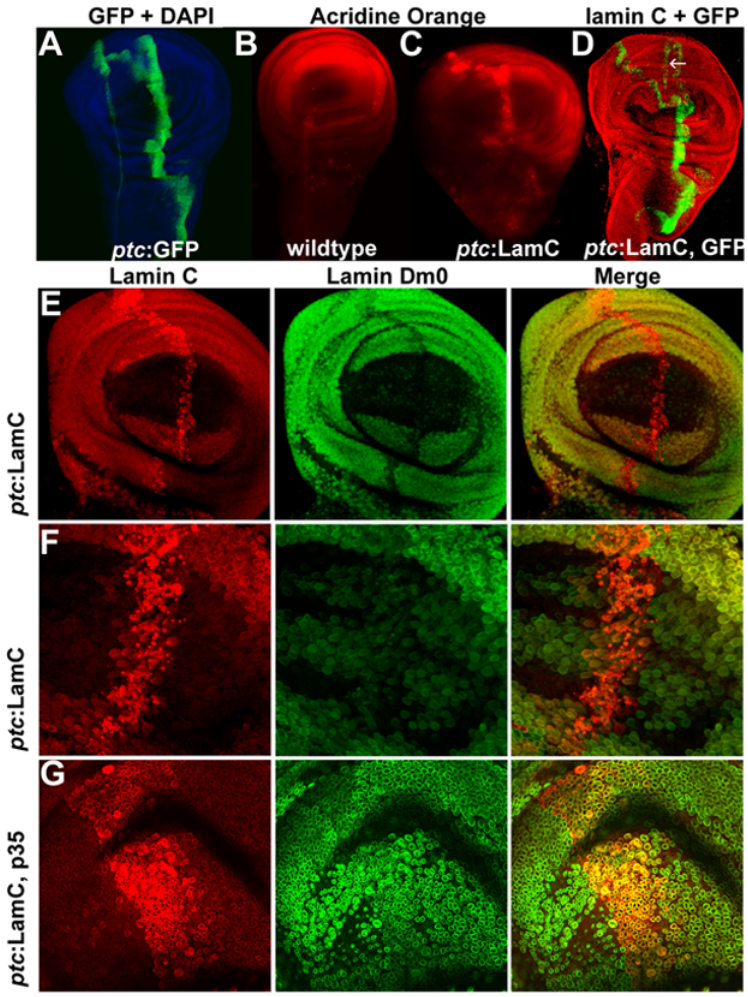


Figure 4. Overexpression of lamin C induces apoptosis in the wing disc. (A) *ptc*-GAL4/UAS-GFP wing disc showing the expression pattern of the GAL4 driver. (B, C) Wild-type (B) and *ptc*-GAL4/UAS-lamin C (C) wing discs stained with acridine orange, which reflects apoptotic cell death. Note, considerable apoptosis all along the A/P boundary, the domain of *ptc*-GAL4 driver. Wild-type wing discs do not show any acridine orange staining. (D) *ptc*-GAL4/UAS-GFP; UAS-lamin C wing disc. Note that the width of the A/P boundary is considerably narrow in this disc compared to the wild-type shown in (A), particularly in the ventral compartment (arrow). (E) *ptc*-GAL4/UAS-lamin C wing discs stained with antibodies to lamin C (red) and lamin Dm_0 (green). (F) Wing pouch of wing disc in (D) is shown at higher magnification. Note, decrease in lamin Dm_0 levels due to overexpression and mislocalization of lamin C. (G) *ptc*-GAL4/ UAS-p35; UAS-lamin C wing disc stained for lamin C (red) and lamin Dm_0 (green). Note restoration of normal width of the A/P boundary and normal levels of lamin Dm_0 due to inhibition of apoptosis by P35.

of gene expression, and has been shown to mediate PEV (reviewed by Weiler and Wakimoto 1995; Eissenberg and Elgin 2000). In the PEV assay, the *In(1)w*^{m4h} also carried the HP1 allele *Su(var)205*. This stock showed maximum suppression of PEV than the *39C-72*. We, therefore, sought to determine the effects of loss-of-lamin C or gain-of-lamin C on the distribution of HP1.

Salivary gland cells of the third instar larva do express LamC (figure 6A). In those cells, intense staining of HP1 is observed only in specific regions near the nuclear periphery (figure 6,B&C). *ptc*-GAL4 is expressed specifically in the salivary glands, but not in the adjacent fat bodies. *ptc*-GAL4-induced expression of *lamC*^{RNAi} in the salivary glands led to more diffused HP1 staining and often at reduced levels (figure 6D). Cells with reduction in HP1 levels showed change in nuclear morphology. Salivary gland nuclei looked small and elongated (figure 6D). In peripodial cells too intense staining of HP1 is observed only in specific regions near

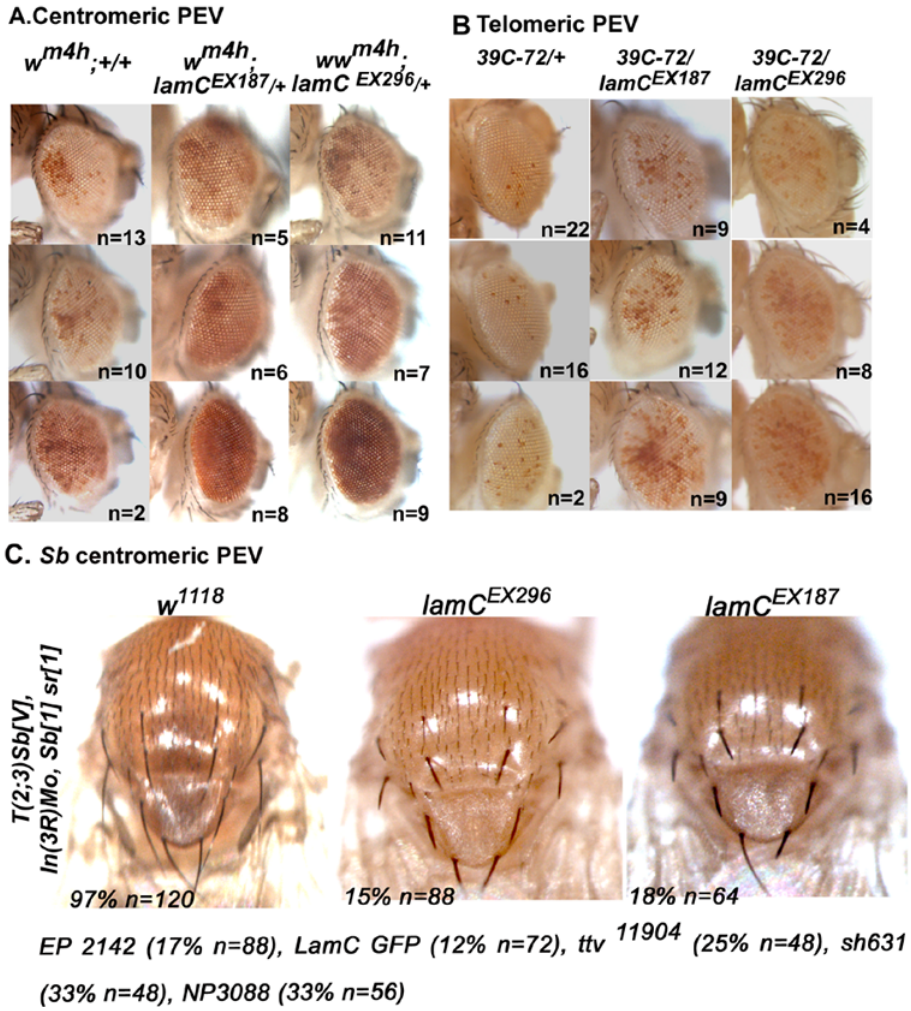


Figure 5. Suppression of PEV by *lamC* mutations. Strains bearing (A) centromeric or (B) telomeric insertions of the *white*$^{+}$ marker were crossed to homozygous w^{1118} (first column), heterozygous $lamC^{EX187}$ (second column) or $lamC^{EX296}$ (third column) alleles. For each cross, three representative eyes are shown. Number (*n*) in each panel represents number of flies examined in one set of experiments. Note suppression of variegation in the background of heterozygous *lamC* alleles. (C) Suppression of PEV by lamC mutations. Flies bearing variegating *Sb* locus with a pericentromeric heterochromatin insertion $T(2;3)Sb^{V}$ (Sinclair *et al*. 1983) were crossed to homozygous w^{1118}, heterozygous $lamC^{EX187}$ or $lamC^{EX296}$ alleles. For each cross, one representative thorax is shown. Number (*n*) in each panel represents number of flies examined in one set of experiments. Due to the translocation of heterochromatin, $T(2;3)Sb^{V}$ flies show normal thoracic bristles. Suppression of this would lead to the appearance of stubble bristles. Note suppression of variegation in the background of heterozygous *lamC* alleles. Data for additional alleles of this locus are also shown.

the nuclear periphery (figure 6E), while peripodial cells of *Ubx*-GAL4/UAS-lamin C larvae showed striking reduction in HP1 staining (figure 6F).

We further examined the effect of lamin C on HP1 localization at the polytene chromosome level. In wild-type polytene chromosomes of the third instar larva, intense staining of HP1 is observed at the chromocentre (figure 7A), which is consistent with earlier reports (Cryderman *et al*. 1999). *ptc*-GAL4-induced expression of $lamC^{RNAi}$ in the salivary glands led to reduction of HP1 staining in polytene chromosomes and de-condensation of the chromatin (figure 7B). Morin *et al*. (2001) have reported a homozygous lethal GFP-trap P-insertion in the *lamC* locus. In this line, we observed GFP staining in aggregates (figure 7C), suggesting mislocalization of lamin C. Interestingly, polytene chromosome preparations from the salivary glands of lamin C::GFP trap line showed significant reduction in HP1 levels (figure 7E). Further, *ptc*-GAL4-induced overexpression of lamin C in the

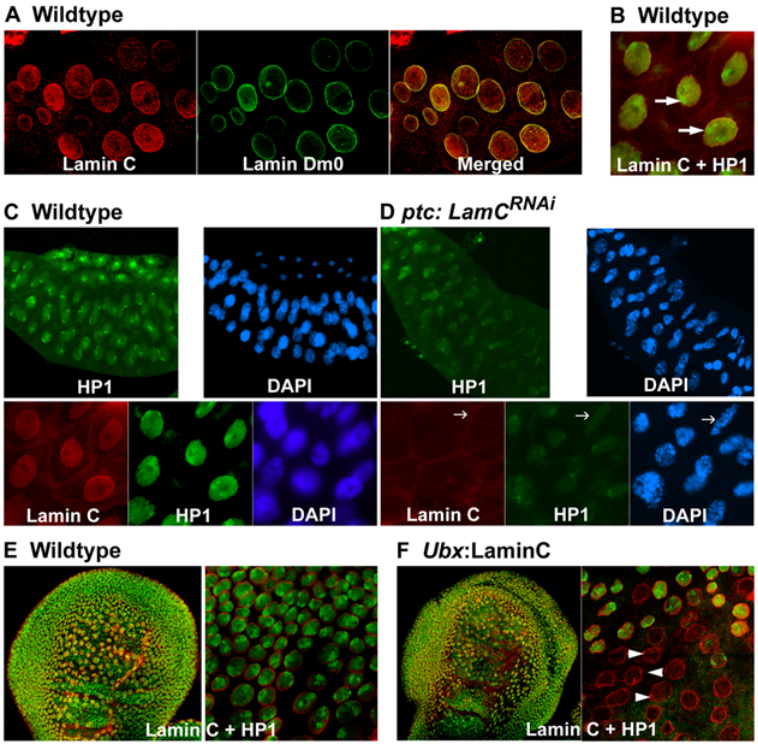


Figure 6. Downregulation of *lamC* affects localization of HP1. (A–C) Wild-type salivary gland cells stained with antibodies to lamin C and lamin Dm_0 (A), lamin C and HP1 (B) and HP1 and DAPI (C) as labelled on the images. Note, HP1 staining is predominantly in one region of the nucleus, near the nuclear periphery (arrows). (D) *ptc*-GAL4/ UAS-*lamC-UTR*^{RNAi}; UAS-*lamC-tail*^{RNAi} salivary glands stained for lamin C (red), HP1 (green) and with DAPI (blue). Note more diffused staining of HP1 suggesting mislocalization (arrows). Few cells also show reduced staining and such cells are smaller and elongated (asterisks in DAPI panel). Panels below C and D, show few cells at higher magnification for better comparison. (E–F) Peripodial cells of wild-type (E) and *Ubx*-GAL4/UAS-lamin C (F) wing discs stained for lamin C (red) and HP1 (green). Note, wherever the peripodial cells show lamin C aggregates, HP1 is either absent or mislocalized (arrowheads in F).

salivary glands also showed drastic reduction in HP1 binding at the chromocentre (figure 7F). Finally, we estimated the levels of HP1 by Western blot analyses and observed that in the context of both overexpression and knock-down of lamin C, HP1 levels are reduced by approximately 2-fold (figure 8). Taken together, these observations suggest that lamin C is required for appropriate chromosome organization and localization of HP1. Disruption in lamin C levels, by either downregulation or upregulation, causes disruption in nuclear lamina, chromosome organization, and distribution of HP1 and thereby causes lethality at the cellular level.

Discussion

Genetic analysis of lamC

The genetic analysis of the *Drosophila lamC* gene is complicated due to its position within the fifth intron of the essential gene *ttv*. Lamin C is not maternally stored and its expression is initiated by 12–15 h of embryonic development. Levels of lamin C protein increase at later stages of embryonic development and remain high from larval stages onwards (Riemer *et al*. 1995). RNAi-mediated downregulation of *lamC* ubiquitously in all cells too resulted in early larval lethality. Tissue-specific downregulation resulted in a variety of phenotypes in wings, legs, thorax and eyes. At the cellular level, we observed defects in nuclear lamina organization and cell death. Thus, it appears that *lamC* is an essential gene required for cell survival, although its requirement appeared to be restricted to postembryonic stages as well as in non-neuronal tissues.

Studies in cultured cells have demonstrated that a number of lamin A/C mutations cause deleterious effects on nuclear structure and functions, including aberrant muscle differentiation (Östlund *et al*. 2001; Raharjo *et al*. 2001; Favreau *et al*. 2004; Mariappan and Parnaik 2005; Manju *et al*. 2006; Parnaik and Manju 2006). Interestingly, majority of

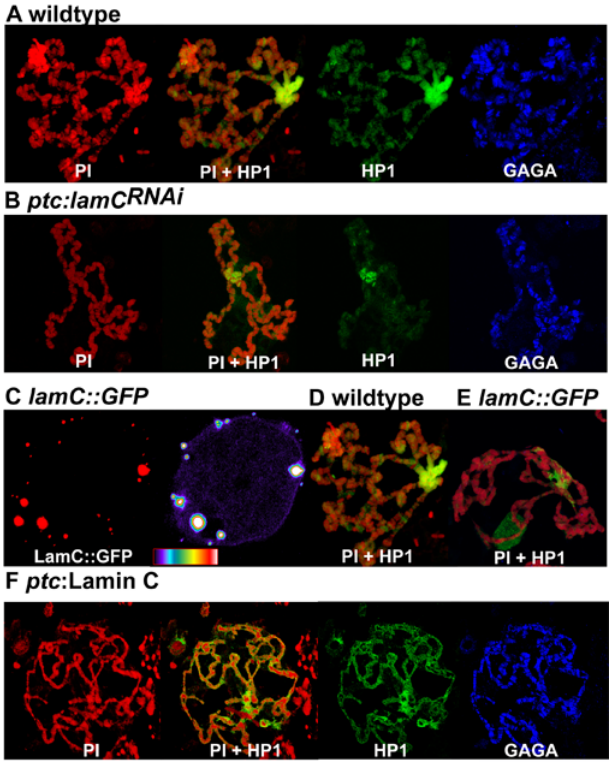
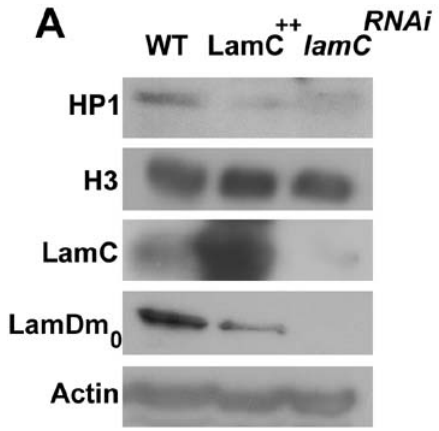


Figure 7. Effect of changes in LamC levels on HP1 localization on polytene chromosomes. (A–B) Wild-type (A) and *ptc*-GAL4/ UAS-*lamC-UTR*^{RNAi}; UAS-*lamC-tail*^{RNAi} (B) polytene chromosomes stained for DNA (with propidium iodide; PI), HP1 (green) and GAGA factor (blue). Note, the preferential localization of HP1 on the chromocentre in wild-type cells, which is significantly depleted when lamin C is downregulated. Expression pattern of GAGA factor is unaltered suggesting that effect of HP1 is specific. Polytene spread is better in B than A, which is why regions of intense PI and GAGA staining are missing in B. (C) Magnified salivary gland cell of a *lamC*::GFP strain, wherein GFP is fused to lamin C. The salivary gland is stained for lamin C (red). Note aggregates of lamin C. The spectral image of a magnified salivary gland cell is shown on the right side, which shows relative levels of *lamC*::GFP. Aggregates of lamin C are evident in this image. (D–E) Wild-type (D) and *lamC*::GFP (E) polytene chromosomes stained for HP1 (green) and DNA (PI). Note, significant depletion of HP1 in E. (F) *ptc*-GAL4/ UAS-lamin C polytene chromosme spreads stained for DNA (with propidium iodide; PI), HP1 and GAGA factor. Note, significant depletion of HP1 from the chromocentre. Expression pattern of GAGA factor is unaltered suggesting that effect of HP1 is specific.



B

	WT	LamC^{++}	*lamC*^{RNAi}
HP1	1.00	0.33±0.06	0.52±0.06
H3	1.00	0.88±0.04	0.59±0.45
LamC	1.00	2.16±0.18	0.16±0.13
LamDm$_0$	1.00	0.59±0.34	0.04±0.00

Figure 8. HP1 and lamin Dm$_0$ levels are reduced in the background of increased or decreased levels of lamin C. (A) Western blot analyses of wild-type (WT), *hs*-GAL4/UAS-LamC (LamC^{++}) and *hs*-GAL4/UAS-*lamC*^{RNAi} (*lamC*^{RNAi}) larvae for HP1, histone 3, lamin C, lamin Dm$_0$ and actin. (B) Quantitative analysis of protein levels expressed as fold change compared to wild-type after normalization to actin (mean ± s.d. from three blots).

lamin A/C mutations express mutant forms of lamins rather than depleting their expression. Furthermore, overexpression of wild-type lamin A/C can also affect nuclear functions and morphology (Kumaran *et al*. 2002; Favreau *et al*. 2003).

In a lone study on the effect of misexpression of lamin C in *Drosophila*, Stuurman *et al*. (1999) overexpressed lamin C using a heat-shock promoter, which resulted in melanotic tumours and larval death. As this gene construct had a frameshift mutation at the 3′ end, which produced 59 unrelated amino acids at the C-terminus, the implications of this data for the functions of wild-type lamin C are not clear. In our studies on tissue-specific overexpression of lamin C, we have observed a variety of phenotypes depending on the tissue and domain of expression, in particular, in dividing cells and in imaginal discs, which contribute to adult structures. Severe disruption of larval and adult muscles occurred upon misexpression of lamin C under muscle-specific enhancers. At the cellular level, we observed distinct aberrations in nuclear lamina morphology as well as deleterious effects on cell survival leading to apoptosis. Interestingly, lamin C was normally incorporated into neuronal cells and did not affect their differentiation. This is consistent with earlier studies showing that ectopically expressed lamin A is assembled at the nuclear periphery in mammalian embryonal cells lacking A-type lamins (Collard and Raymond 1990; Horton *et al*. 1992) and does not interfere with their capacity to differentiate into neuronal cells (Peter and Nigg 1991).

Taken together, it is evident that both downregulation and upregulation of *lamC* have similar phenotypes, thereby underlining the importance of precise assembly of lamin C into the nuclear lamina. However, it is not yet clear if the lethality caused by downregulation and upregulation of lamin C is through a common mechanism.

Importance of lamins in chromatin organization

Binding of lamins to chromatin is well documented *in vivo* and *in vitro* experiments (reviewed by Goldman *et al*. 2002). Lamins are involved in the organization of RNA polymerase II transcription in the nucleus (Kumaran *et al*. 2002; Spann *et al*. 2002). In addition, lamins can bind to transcriptional regulators such as pRb (Ozaki *et al*. 1994) and SREBP1 (Lloyd *et al*. 2002) and might be required for specific gene regulation. Hutchinson–Gilford progeria disease is caused by mutations in human *laminA* that affect the processing of lamin A precursor, which in turn cause widespread alterations in nuclear structure and loss of heterochromatin (Goldman *et al*. 2004). Further reports also suggest that the human cells expressing mutant forms of lamins show nuclear dysmorphism and chromatin disorganization (Columbaro *et al*. 2005) and display loss of heterochromatin and epigenetic markers (Shumaker *et al*. 2006). Interestingly, a comprehensive survey of genome organization in *Drosophila* has indicated that ~500 genes interact with lamin Dm_0 in embryonic cells (Pickersgill *et al*. 2006).

Our data on the suppression of PEV by mutant *lamC* alleles is consistent with a role for the nuclear lamina in chromatin organization. Although the exact mechanism of silent chromatin spreading is not clear, several factors have been shown to be involved in this process (Weiler and Wakimoto 1995; Wallrath 1998). Of these, HP1 is important for inducing heterochromatic gene silencing (Danzer and Wallrath 2004). Our results demonstrate that downregulation or misexpression or aberrant localization of lamin C leads to depletion of HP1 from the chromatin. This suggests that HP1 localization is dependent on the proper organization and function of the nuclear lamina. In addition to mediating gene silencing, HP1 promotes accurate chromosome segregation in *Drosophila* embryos (Kellum and Alberts 1995). Further, several genes are misregulated in HP1-depleted mutants (Cryderman *et al*. 1999). Loss of HP1 upon lamin C mislocalization might also lead to misregulation of gene expression, causing aberrant cell division and ultimately cell death.

In *Drosophila*, interphase centromeres and telomeres tend to cluster at the nuclear periphery and lamina (reviewed by Pluta *et al*. 1995). The PEV suppressor *Su(var)2-10* is essential for chromosome structure and inheritance, and is involved in nuclear organization (Hari *et al*. 2001). Inhibition of *Su(var)2-10* function disrupts telomere–telomere and telomere–lamina interactions. *Su(var)2-10*, which encodes a PIAS protein homolog, partly colocalizes with the peripheral lamina and is also detectable in the interior of the nucleus. Although there is substantial evidence for the presence of lamins in the interior of the mammalian cell nucleus (Moir *et al*. 1994; Hozak *et al*. 1995; Jagatheesan *et al*. 1999; Moir *et al*. 2000), internal lamins have not been detected in *Drosophila* nuclei. It is possible that lamin C bridges the nuclear lamina with chromatin by interacting with specific binding proteins. Further investigation on the lines of yeast two-hybrid screens and other similar techniques may reveal the precise molecular mechanism by which lamin C functions in the nucleus.

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