CHAPTER 5

Lamin C in

Chromatin Organisation
5.0 Introduction

In late telophase of mitosis, lamins move into the nucleus and are assembled into the newly formed nuclear envelope. Earlier studies have revealed that the lamins are constituents of the inner nuclear membrane (INM) and bind to DNA, histones and chromatin (Goldman et al., 2002). The inner nuclear membrane proteins with the LEM domain such as lamin B receptor (LBR), emerin and LAP2β bind to chromatin through a protein – Barrier-to-Autointegration-Factor (BAF). These proteins have also been suggested to have a role in higher order chromatin structure as well as in nuclear assembly (Zastrow et al., 2004). The INM protein LBR shows interactions with histone H3/H4 and Heterochromatin protein1 (HP1) (Polioudaki et al., 2001). The mouse knockout for Lamin A/C has shown reduction in heterochromatin (Sullivan et al., 1999). Lamin A/C is also known to interact with transcriptional factors such as SREBP1 and pRB (Ozaki et al., 1994; Lloyd et al., 2002).

The nucleus is organized into specific compartments. The chromosomes are known to occupy defined positions in the nucleus referred to as chromosome territories. It has been observed that heterochromatin and associated proteins preferably associate with the nuclear periphery. This is reflected in the observation that the Drosophila gypsy insulator insertion (which heterochromatinizes the region of insertion) is known to localize flanking genes to the nuclear envelope (Gerasimova and Corces, 1998). The localization of specific genes at the nuclear periphery may lead to transcriptional silencing, which has been observed in studies on telomeric silencing in yeast (Taddei et al., 2004). On the other hand, the gene rich regions are positioned in the interior of the nucleus (Henikoff, 2000; Henikoff et al., 2000).

As Lamin C is a determinant of nuclear envelope integrity and vital functions of the cell, it is possible that loss of Lamin C function could lead to changes in global gene expression leading to several phenotypes. Towards investigating this, we have made an attempt to understand the role of Lamin C in chromatin organization and gene expression using genetic assays.
5.1 *lamC* alleles suppress Position Effect Variegation

The *white* (*w*) gene in *Drosophila* is a very good marker to study changes in gene expression due to chromatin organization and structure. Transgene flies for *white* in the background of loss of function of endogenous *white* gene show various levels and patterns of expression depending on the site of insertion in the genome. If the *white* gene is inserted in the vicinity of the centromeric region, it is not expressed due to the repressive influence of the centromeric heterochromatin (Reuter and Wolff, 1981). At the phenotypic levels, within the eye of a transgenic fly, some ommatidia show red pigment and some do not show. Such variegated eye colour phenotypes constitute a good assay system to study influence of chromatin organization on gene expression. This assay is commonly known as Position Effect Variegation (PEV) assay. PEV has been attributed to cis-spreading of the heterochromatic state and is influenced by chromatin structure and nuclear organization (Walrath and Elgin, 1995; also reviewed by Walrath, 1998). Genes associated with PEV have been identified by analysis of suppression of PEV (reviewed by Weller and Wakimoto, 1995). When heterochromatinization is removed due to any mutations such as loss of function mutations in *HP1*, *w*<sup>+</sup> gene expression is restored (Figure 5.1A). Such mutations are called suppressor of PEV or *Su(var)*. Here we have investigated the effect of mutations in *lamC* on PEV at centromeric and telomeric heterochromatin. We used the *w*<sup>+</sup> transgene *Wm4h* to examine the effect of *lamC* on centromeric PEV and 39C72 insertion to test effects on telomeric PEV.

The *lamC* excision alleles, *lamC<sup>ex06</sup>* and *lamC<sup>ex09</sup>* were crossed to *Wm4h* and 39C72. They were also crossed to *w<sup>118</sup>* lines as a control. The progeny of all the crosses were scored for the modification of PEV. A significant number (for statistics, see numbers in Figure 5.1) of double heterozygous flies had nearly normal red eye colour with little evidence of variegation, in contrast to the variegated eyes of the PEV strains crossed to *w<sup>118</sup>* strains (Figure 5.1 C-D) and quantitative analysis of eye pigment showed a 2.0 to 2.5 fold increase in pigment levels (Figure 5.1 B). This suppression of PEV by heterozygous *lamC<sup>ex09</sup>* and *lamC<sup>ex06</sup>* mutants suggests de-condensation of chromosomal organization in both
In(1) \textsuperscript{W\textsuperscript{4h}} The White gene is relocated to heterochromatin by paracentric inversion.

**C Centromeric PEV**

- \textsuperscript{wM4h/+}
- \textsuperscript{wM4h/lam\textsuperscript{ex09}}
- \textsuperscript{wM4h/lam\textsuperscript{ex06}}

**D Telomeric PEV**

- \textsuperscript{39C-72/+}
- \textsuperscript{39C-72/lam\textsuperscript{ex09}}
- \textsuperscript{39C-72/lam\textsuperscript{ex06}}

**Figure 5.1 Suppression of PEV by lamC mutations.**

(A) Schematic showing the PEV in white gene. The White gene expression (required for red eye colour) is repressed when it is moved into the proximity of a heterochromatic region- here Centromere (eye colour appears mosaic).

(B) Strains bearing (B) centromeric or (C) telomeric insertions of the white transgene were crossed to homozygous \textsuperscript{W\textsuperscript{1118}} (first column), heterozygous \textsuperscript{lam C\textsuperscript{ex09}/+} (second column) or lam C\textsuperscript{ex06}/+ (third column) alleles. The spectrophotometric estimation of eye pigmentation was carried out on 10 flies of each genotype. There is an average \( \sim 2.5 \) fold increase in expression of eye pigment in experimental crosses compared to control flies.

(C-D) Three representative eyes of the above-mentioned 6 crosses. 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.
centromeric and telomeric loci and thereby provides evidence for a role for Lamin C in chromatin organization.

5.2 Lamin C::GFP protein trap shows abnormal nuclear envelope organization and depletion of Heterochromatin protein 1

The nuclear envelope organization in Drosophila salivary gland nuclei appears as a uniform rim (Figure 5.2A), when stained with Lamin C specific antibodies. The Lamin C::GFP protein trap G-158 has a pPTT transposase inserted in the first intron making an in frame GFP fusion with rod domain of endogenous Lamin C protein. This is a homozygous lethal insertion and is also an allele of ttv. As rod domain is required for the dimerization of the lamin filaments, the internal GFP fusion is likely to disrupt the dimerization and thereby may affect proper assembly of the nuclear lamina. Indeed, Lamin C::GFP is localized as aggregates in the nuclear envelope (Figure 5.2B).

The aberrant nuclear structure in Lamin C::GFP nuclei is likely to affect the chromatin organization. Drosophila HP1 is a chromatin binding protein known to be associated with the chromocentre and also the termini of chromosomes. In wild-type salivary gland cells (we used salivary gland cells for this purpose as the nuclei are much bigger and it is easier to examine internal details) of the third instar larva, intense staining of HP1 is observed only in specific regions near the nuclear periphery (Figure 5.3 A.), probably at the chromocentre (Figure 5.2 C), which is consistent with earlier reports (Cryderman et al., 1999). We examined the levels of HP1 in salivary gland polytene spreads in heterozygous Lamin C::GFP animals. The levels of HP1 were relatively lower in heterozygous Lamin C::GFP compared to wild-type suggesting changes in chromatin organization due to compromised Lamin C assembly (Figure 5.2 C).

5.3 Studies on nuclear organization in lamC' background

Above-mentioned results with Lamin C::GFP suggest a role for Lamin C in HP1 localization. As this could be a neomorphic phenotype, we further examined this phenomenon by down-regulating Lamin C levels in salivary gland cells using
Figure 5.2 The *pPTT* LaminC::GFP protein trap makes aberrant Lamin C-GFP fusion protein forming nuclear envelope aggregates and affects localization of HP1.

A. The *pPTT* LaminC::GFP, a protein trap insertion in the first intron, makes a fusion with Lamin C at the rod domain.

B. Salivary gland cells of a LaminC::GFP strain stained for GFP and Lamin C. The spectral image of a magnified salivary gland cell is shown on the right side. Aggregates of Lamin C are evident in this image.

C. Polytene chromosome preparations from wild-type and LaminC::GFP strains immunostained for HP1. Note significant down-regulation of HP1 in LaminC::GFP.
Figure 5.3. Down-regulation of lamC affects sub-nuclear localization of HP1.

(A) Wild type salivary gland cells stained with antibodies to Lamin C, Lamin Dm0 and HP1 as labeled on the images. Note specific intense staining of HP1 in different parts of the nucleus.

(B) ptc-GAL4/UAS-GFP salivary gland stained for both GFP (green) and with DAPI (blue) showing the expression pattern of the GAL4 driver in the salivary gland. Note ptc-GAL4 driver is not expressed in the fat bodies.

(C) Wild type salivary gland cells stained with HP1 (C) and DAPI (C'). Note, HP1 staining is predominantly in one region of the nucleus.

(D-F) ptc-GAL4/UAS-lamC-UTR^{RNAi}; UAS-lamC-tai^{RNAi} salivary glands stained for Lamin C (D), HP1 (E) and with DAPI (F). Note down-regulation and mis-localization (more diffused localization) of HP1. In all such nuclei the DAPI-stained DNA appears more elongated.

(G-H) High magnification image of wild type (G) and ptc-GAL4/UAS-lamC-RNAi^{UTR}; UAS-lamC-RNAi^{tail} (H) salivary gland cells stained for Lamin C (red), HP1 (green) and with DAPI (blue). Note in wild type HP1 is highly localized (probably in the chromocentre) and the staining appears very intense. In cells wherein Lamin C levels are down-regulated, HP1 staining is more diffused and thereby less intense.
Lamin C in chromatin organization

RNAi method. The ptc-GAL4 was identified as a strong driver to express a transgene under UAS promoter in salivary gland cells amongst Kr-GAL4, fkh-GAL4, ftz-GAL4, eagle GAL4, hs-GAL and sgs-GAL4 drivers. ptc-GAL4 shows strong and continuous expression during larval stages in salivary gland and is absent in adjacent fat body cells.

We examined the pattern and levels of HP1 expression in salivary gland cells depleted of Lamin C using Lam CRNAi transgene. We expressed two copies of RNAi to bring Lamin C levels to almost null using ptc-GAL4 driver (Figure 5.3A). We observed considerable reduction in the levels of Lamin C in large number of salivary gland cells and nuclei of those cells were longer and more elongated than nuclei of wild type cells. We also observed severe reduction in the levels of HP1 at chromocentre indicating loss of heterochromatinization (Figure 5.3B). None of these changes/phenotypes were observed in the adjacent fat-body cells, wherein ptc-GAL4 driver is not expressed, suggesting specificity of these phenotypes to depletion of Lamin C. The change in nuclear morphology could be due to loss of the pericentric heterochromatinization. DAPI staining of salivary gland nuclei also suggested more diffused chromatin compared to condensed chromatin in the nucleus (Figure 5.3 C). All these phenotypes point to a role for Lamin C in chromatin condensation and HP1 organization.

5.4 HP1 is mis-localized in cells over-expressing Lamin C

We over-expressed Lamin C in salivary glands using ptc-GAL4, which caused severe reduction in the gland size. The nuclear envelope showed aggregates of Lamin C and Lamin Dm0 (Figure 5.4A-C), a phenotype similar to Lamin C::GFP. Interestingly, much of the over-expressed protein appeared to be in some form of vesicles around the nucleus, suggesting abnormal assembly of lamina due to inability of vesicle fusion. Such aggregates are observed in human cells expressing disease-causing mutant forms of Lamin A/C proteins (Hubner et al., 2006). Interestingly, we observed complete mis-localization of HP1 from chromocentre to the nuclear periphery in such salivary gland cells (Figure 5.4 D and G). In wing disc peripodial cells (which are diploid) over-expressing Lamin C,
we observed loss of HP1 in the nuclei (Figure 5.4 H-1), ruling out the possibility that the observed phenotypes in salivary gland cells are due to some unusual modification of polyploid cells. Taken together, these results indicate possible direct interaction between nuclear envelope proteins such as Lamin C and HP1 during the nuclear envelope assembly.

5.5 Discussion

Lamins are the components of a filamentous network underlying the inner nuclear membrane termed the nuclear lamina. 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 (reviewed in Stuurman et al. 1998). Lamin B (Belmount et al., 1993; Moir and Goldman 1993) and Lamin A/C (Moir et al., 1994; Hozak et al., 1995; Jagatheesan et al., 1999) have also been identified in the interior of the nucleus. The lamins and lamin-associated proteins are known to bind chromatin. The LEM binding domain proteins such as LBR, emerin and LAP2 are known to bind chromatin through BAF (Cai et al., 2001; Laguri et al., 2001; Wolff et al., 2001). The lamin B receptor is known to bind to the non-histone protein HP1 (Ye and Worman 1996). Mutations in LBR show defects in HP1 localization and loss of LBR-HP1 association results in dissociation of LBR, leading to destabilization of the nuclear envelope (Okada et al., 2005).

Vertebrate cells expressing mutant lamins have also shown loss of epigenetic markers. HGPS cells show alteration of nuclear structure and heterochromatin (Goldman et al., 2004). The HGPS cells expressing mutant Lamin A (LADA50) show loss of facultative and constitutive heterochromatin marker H3K27 methylation and H3K9 trimethylation - a pericentric heterochromatin marker (Shumaker et al., 2006). In the present study we have provided evidence for the involvement of Lamin C in the localization of heterochromatin protein HP1 and consequent effects on gene expression (on PEV). Thus, our results suggest that loss of nuclear envelope organization has a profound influence on protein-protein interactions during nuclear envelope assembly.
Figure 5.4: The Effect of Over-expression of Lamin C on the localization of Lamin Dm0 and HP1.

(A-B) High magnification image of single salivary gland cell of ptc-GAL4/UAS-Lamin C larva stained for Lamin C (red; A) and Lamin Dm0 (green; B). Note rough, vesicular staining patterns of Lamin C and Lamin Dm0 compared to smooth staining in wild type salivary gland cells. These vesicles show colocalization of Lamin C and Lamin Dm0. (D) The wild type nucleus shows normal HP1 binding to chromocentre. (E) High magnification image of single salivary gland cell of ptc-GAL4/UAS-Lamin C larva stained for HP1 (green) and with DAPI (blue). Note mislocalization of HP1 and decondensed chromatin. It appears that over-expressed Lamin C has caused localization of HP1 to the nuclear periphery. (F-G) Peripodial cells of wild type (E) and Ubx-GAL4/UAS-LaminC wing discs stained for Lamin C (red) and HP1 (green). (H-I) High magnification images of discs shown in F and G, respectively. Note wherever the peripodial cells show Lamin C aggregates, HP1 is either absent or mislocalized, arrow in (I).
PEV reflects gene-silencing due to heterochromatinization. HP1 is known to induce heterochromatinization dependent or independent of histone methyl transferase (Suvar-309), which causes H3K9 trimethylation on nucleosomes and helps in the spreading of heterochromatin. In all these situations, the localization of HP1 shows the hallmarks of heterochromatin state of the chromosomes. The Suvar-210 codes for PIAS protein homologue, which shows partial co-localization with nuclear lamina. Inhibition of Suvar-210 is known to disrupt telomere-telomere lamina interactions. The chromosomal deletion that uncovers lamC shows suppression of telomeric position effect (Donaldson et al., 2002). In our study the lamC mutations have shown suppression of PEV in both centromeric and telomeric loci, suggesting the influence of Lamin C on heterochromatic regions at centromeric and telomeric regions.

The depletion or mis-localization of HP1 can result in changes in higher order structure of chromosomes and gene function. HP1 is known to bind various specific sites in the centromeric and telomeric regions. HP1 is also known to localize in more than 200 sites throughout euchromatin (James et al., 1989; Fanti et al., 2003). The HP1 mutant animals showing reduced protein levels affect expression of 284 genes, suggesting a role for HP1 in global gene expression. These include genes controlling cell death. The effect of loss of HP1 protein on cell death genes may be due to indirect effects in the change in global expression (Cryderman et al., 2005). Thus, lethality observed in loss- and gain-of-function background of Lamin C could be attributed to down-regulation of HP1 and consequent changes in gene expression patterns. The mutations in human Lamin A/C show a number of disease phenotypes. These could be due to global changes in gene expression.