The role of Drosophila Lamin C in muscle function and gene expression

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SUMMARY
The inner side of the nuclear envelope (NE) is lined with lamins, a meshwork of intermediate filaments that provides structural support for the nucleus and plays roles in many nuclear processes. Lamins, classified as A- or B-types on the basis of biochemical properties, have a conserved globular head, central rod and C-terminal domain that includes an Ig-fold structural motif. In humans, mutations in A-type lamins give rise to diseases that exhibit tissue-specific defects, such as Emery-Dreifuss muscular dystrophy. Drosophila is being used as a model to determine tissue-specific functions of A-type lamins in development, with implications for understanding human disease mechanisms. The GAL4-UAS system was used to express wild-type and mutant forms of Lamin C (the presumed Drosophila A-type lamin), in an otherwise wild-type background. Larval muscle-specific expression of wild type Drosophila Lamin C caused no overt phenotype. By contrast, larval muscle-specific expression of a truncated form of Lamin C lacking the N-terminal head (Lamin CΔN) caused muscle defects and semi-lethality, with adult ‘escapers’ possessing malformed legs. The leg defects were due to a lack of larval muscle function and alterations in hormone-regulated gene expression. The consequences of Lamin C association at a gene were tested directly by targeting a Lamin C DNA-binding domain fusion protein upstream of a reporter gene. Association of Lamin C correlated with localization of the reporter gene at the nuclear periphery and gene repression. These data demonstrate connections among the Drosophila A-type lamin, hormone-induced gene expression and muscle function.

KEY WORDS: Drosophila, Gene expression, Lamins, Larval muscle, Nuclear envelope

INTRODUCTION
The nuclear lamina is a protein meshwork that lines the inner nuclear membrane. Type V intermediate filament proteins called lamins and membrane-associated proteins, many of which possess a LEM domain (named for founding members LAP2, Emerin and MAN1), make up the lamina (Cai et al., 2001; Herrmann and Aebi, 2004; Lin et al., 2000; McKeon et al., 1986; Pinto et al., 2008; Schulze et al., 2009; Stuurman et al., 1998; Wolff et al., 2001). Lamins have a conserved N-terminal globular head domain, a central coiled rod domain and a C-terminal tail that includes an Ig-fold structural motif (Fisher et al., 1986; McKeon et al., 1986). Lamins play roles in nuclear shape determination, nuclear positioning, chromosomal arrangement, heterochromatin organization, nuclear pore function, chromosome segregation, cytoskeletal organization and signal transduction (Capco and Penman, 1983; Furukawa and Hotta, 1993; Liu et al., 2000; Morris, 2003; Paddy et al., 1990; Schirmer et al., 2003; Starr and Fischer, 2005; Sullivan et al., 1999; Wilhelmsen et al., 2006).

Lamins are classified as A- and B-types (Burke and Gerace, 1986; Georgatos et al., 1994; Gerace and Blobel, 1980; Gerace and Burke, 1988; Riemer et al., 1995; Stuurman et al., 1998). A-type lamins are expressed in nearly all differentiated somatic cells and are not essential for cell viability (Fisher et al., 1986). By contrast, B-type lamins are ubiquitously expressed and are essential for cell viability (Biamonti et al., 1992; Harborth et al., 2001; Pollard et al., 1990). Mutations in LMNA, the gene encoding A-type lamins in humans, give rise to at least 12 disorders termed laminopathies, including autosomal dominant Emery-Dreifuss muscular dystrophy (AD-EDMD) (Bonne et al., 1999). Laminopathies have tissue-specific phenotypes, despite the global expression of LMNA. EDMD is also caused by mutations in the gene encoding emerin, a LEM domain protein (Bione et al., 1994; Manilal et al., 1996; Nanego et al., 1996). Emerin interacts with A-type lamins (Clements et al., 2000; Sakaki et al., 2001) and is mislocalized in many patients with LMNA mutations (Ben-Harush et al., 2004; Muchir et al., 2003; Sullivan et al., 1999).

Here, we focus on the role of the N-terminal head of A-type lamin with respect to muscle function and gene expression. Structural studies implicate the head domain in lamina assembly (Herrmann and Foisner, 2003; Isobe et al., 2007; Sasse et al., 1998; Shumaker et al., 2005; Stuurman et al., 1996). Lamins dimerize through the rod domain and form head-to-tail interactions to generate filaments (Sasse et al., 1998; Stuurman et al., 1996). In humans, a mutation in the LMNA gene, which is presumed to produce a truncated protein lacking the head domain, has been implicated in a form of EDMD possessing a neurological phenotype (Walter et al., 2005).

We have developed Drosophila melanogaster as a model to study tissue-specific functions of A-type lamins. Drosophila is the only invertebrate model organism known to possess the two types of lamins found in humans (Klapper et al., 1997; Riemer et al., 1995; Schulze et al., 2005). The Drosophila genome possesses two lamin genes: Lamin C, proposed to encode the A-type lamin, and Lamin Dm0, the B-type lamin (Riemer et al., 1995). Expression of mutant forms of Lamin C that alter the N- or C-terminal domain associate with the nuclear envelope (NE) and, in some cases, cause...
lethality when expressed in larval muscle but not in other tissues (Schulze et al., 2009). By contrast, alterations in the rod domain cause abnormal lamin aggregation within the nucleus but not lethality. Collectively, our studies support a role for the N-terminal head and C-terminal globular domain in larval muscle function.

To better understand the role of the N-terminal head domain, we generated transgenic flies expressing a mutant form of Lamin C lacking the first 42 amino acids (Lamin C ΔN) (Schulze et al., 2009). This mutant form localizes to the NE and causes semi-lethality when expressed in muscle but not in non-muscle tissues (Schulze et al., 2009). Here, we demonstrate that lethality is associated with larval body wall muscle defects that include nuclear and cytoplasmic abnormalities. ‘Adult escapers’ expressing Lamin C ΔN have leg defects that are consistent with a loss of muscle function and disruptions in edcsyne hormone signaling. Collectively, these studies demonstrate a role for the N-terminal head domain of Lamin C in muscle function and gene expression.

MATERIALS AND METHODS

Drosophila stocks and genetic analyses

Drosophila stocks were raised at room temperature on standard sucrose and cornmeal medium (Shaffer et al., 1994). Generation of transgenic stocks expressing wild type and Lamin C ΔN was previously reported (Schulze et al., 2005; Schulze et al., 2009). The GAL4-UAS system (Brand and Perrimon, 1993; Duffy, 2002) was used to drive expression of wild type Lamin C and Lamin C ΔN in specific tissues. The GAL4 driver stocks used in this study are described in Table 1.

Immunostaining of muscles

Larval muscle preparations were performed according to published procedures (Budnik et al., 1990). After fixation, the dissected body wall preparations were blocked in PBS2+ (130 mM NaCl, 7 mM Na2HPO4, 3 mM NaH2PO4, 10 mM EGTA, 0.1% Triton-X 100) containing 0.1% BSA for 1 hour. Muscle preparations were stained with 66 nM fluorescent Phalloidin (Alexa Fluor 488 from Molecular Probes) in PBS2+ containing 0.1% BSA, rat anti-tyrosinated tubulin (1:50 dilution), rabbit anti-D-Titin (anti-52, 1:5000 dilution; a generous gift from Deborah J. Andrew) (Fabian et al., 2007) or rabbit anti-Mlp84b (B54, 1:100 dilution; a generous gift from Deborah J. Andrew) (Fabian et al., 2007). Preparations were blocked in PBS 2+ containing 0.1% BSA for 1 hour incubation at 65°C for 10 minutes. cDNA synthesis was performed using a 78 μl reaction mixture containing 39 μl of 2× SYBR Green PCR Master Mix (Applied Biosystems) and 1 mM of each primer for triplicate reactions in a 25 μl volume per well. Triplicate reactions were performed and averaged. The reaction was first incubated at 95°C for 3 minutes, followed by 40 cycles at 95°C for 30 seconds and 60°C for 1 minute. The sequences for the RT-PCR are shown in Table S1 in the supplementary material. At least three independent experiments were performed for each primer set on three independent RNA samples. Relative levels of mRNA expression were calculated using the cycle threshold (CT) method (Valasek and Repa, 2005). Individual expression values were normalized by comparison with rp49 mRNA.

Immunohistochemical staining of salivary glands and imaginal disc nuclei

Larvae were raised at room temperature in vials, administered a 45 minute heat-shock at 37°C and allowed to recover for 2 hours. Salivary glands and imaginal disc tissues were dissected (the total dissection time for an experiment was less than 1.5 hours), placed in wells and fixed with 2% formaldehyde for 15-20 minutes, followed by three washes of 5 minutes each in PBS2+. Tissues were blocked in PBS2+ containing 0.1% BSA for 1 hour and incubated overnight with primary antibodies at 4°C. After three 15 minute washes with PBS2+, tissues were incubated for 2 hours with the relevant secondary antibodies in the dark at room temperature. Tissues were washed three times for 10 minutes in PBS2+ and mounted in Vectashield H-1000 or Vectashield with DAPI (Vector Laboratories, Burlingame, CA, USA). The primary antibodies used were: mouse anti-Lamin C (LC28.26, 1:500 dilution; University of Iowa Hybridoma Core Facility, IA, USA), mouse anti-LacI (clone 9A5, 1:200 dilution; Upstate Biotechnology), rabbit anti-LacI (1:200 dilution; Stratagene).

Fluorescent in situ hybridization

Salivary glands and diploid tissues were dissected in 1× PBS and fixed in 4% formaldehyde in 1× PBS. The tissue was washed with 2× SSCT (0.3 M NaCl, 0.03 M sodium citrate and 0.1% Tween-20) and treated with RNase A in 2× SSCT for 30 minutes. The tissues were washed with gradually increasing (20%, 40% and 50%) formamide concentration in 2× SSCT at 37°C. Then the tissues were incubated with 100 ng of a DNA fragment representing the lac operator repeats (gift of Y. Rong, NIH) in hybridization solution (10% dextran sulfate, 3× SSC, 50% formamide) at 92°C for 2 minutes, then at 37°C overnight. The tissues were washed with gradually decreasing (50%, 40%, 20%) formamide concentration, counterstained with anti-LacI antibodies and DAPI, and mounted on to glass coverslips.

RESULTS

Expression of Lamin C ΔN causes nuclear organization and muscle defects

In previous studies, we demonstrated muscle sensitivity to mutant forms of Drosophila A-type lamin (Schulze et al., 2009). Here, we extend these studies by testing the effects of expressing Lamin C ΔN in additional tissues and stages of development, with emphasis on muscle. Expression of wild-type Lamin C caused no aberrant morphological phenotype or loss of viability for all drivers tested (Table 1). By contrast, expression of Lamin C ΔN caused semi- or complete lethality when expressed by the larval muscle drivers tested but not by adult muscle or non-muscle drivers (Table 1). Lethality was attributed to the loss of the head domain and not overexpression; western analysis showed that wild-type Lamin C and Lamin C ΔN were expressed at nearly identical levels (see Fig. S1 in the supplementary material). These data demonstrate the importance of the head domain in larval muscle.

To investigate the mechanism by which Lamin C ΔN caused lethality, third instar larval body wall muscles were fixed and stained with antibodies to Lamin C. Expression of wild-type Lamin
C by the C57 larval muscle-specific driver resulted in proper localization of Lamin C (both endogenous and exogenously expressed) at the nuclear periphery of normal shaped nuclei (Fig. 1A). By contrast, up to 13% of the nuclei (n=300) in larvae expressing Lamin C ΔN by the C57 driver exhibited Lamin C aggregation, misshapen and elongated nuclei in which the chromatin appeared to be highly condensed by DAPI staining (Fig. 1A). Given the disruption in A-type lamin localization, we examined the distribution of the nuclear pore complexes (NPCs; Fig. 1B). Larvae expressing wild-type Lamin C showed the anticipated rim pattern of staining around the nucleus (Fig. 1B). By contrast, nuclei of larvae expressing Lamin C ΔN showed NPC aggregation that was enriched at one side of the nucleus in 9% of the nuclei (n=300; Fig. 1B). Lamin proteins interact with LEM domain proteins; therefore, we investigated the localization of Otefin, a potential homolog of mammalian emerin. Larval muscle expressing wild-type Lamin C stained with anti-Otefin antibodies showed peripheral staining around the nucleus. By contrast, the muscle cells of larvae expressing Lamin C ΔN showed aggregation of Otefin in 11% of the nuclei (n=300; Fig. 1C). Lamin proteins interact with the nuclear envelope proteins containing SUN domains (Haque et al., 2006; Hasan et al., 2006; Mejat et al., 2009); therefore, we examined the localization of Klaroid, a Drosophila SUN domain protein (Kracklauer et al., 2007). Klaroid localized to the nuclear periphery in larval muscle nuclei expressing wild-type Lamin C (Fig. 1D). In larval muscle expressing Lamin C ΔN, nuclear aggregates of Klaroid were apparent in 12% of the nuclei (n=300). In addition, atypical cytoplasmic localization was observed (Fig. 1D). Thus, the localization pattern of nuclear proteins belonging to different classes was disrupted upon expression of the headless A-type lamin.

In mammals, SUN domain proteins interact with nesrin proteins within the perinuclear space (Padmakummar et al., 2005). Nesprins extend into the cytoplasm and associate with cytoskeletal components such as actin (Crisp et al., 2006). The mislocalization of Klaroid upon expression of Lamin C ΔN suggested the possibility that cytoplasmic connections might be disrupted. Therefore, body wall muscle from larvae expressing wild-type Lamin C and Lamin C ΔN were stained with antibodies to tyrosinated tubulin, which recognizes newly incorporated tubulin. In the case of wild-type Lamin C, a dense meshwork of microtubules was detected around the nucleus (Fig. 2). This localization pattern is similar to that observed in non-transgenic larvae (data not shown). By contrast, muscle from larvae expressing Lamin C ΔN showed an absence of tyrosinated tubulin meshwork around the misshapen nuclei (n=144; Fig. 2). Given these alterations in tubulin localization, we assayed the organization of actin using Phalloidin, which recognizes filamentous actin. Larvae expressing wild-type Lamin C showed a typical striated muscle fiber pattern expected of Phalloidin staining, whereas ~21% of the muscles in larvae expressing Lamin C ΔN showed disruptions in the striated pattern (n=180; Fig. 2). In addition, Phalloidin staining fibers were observed in ~27% of the nuclei of the Lamin C ΔN expressing larvae (n=157; Fig. 2), suggesting the presence of actin filament formation in the nucleus, similar to those observed in Lamin C null mutants (Schulze et al., 2009). Such fibers were not apparent in nuclei expressing wild-type Lamin C. These data are consistent with Lamin C regulating actin polymerization in the nucleus, a hypothesis supported showing that lamin bundles actin in vitro (Simon et al., 2010).

The presence of intranuclear actin filaments in larvae expressing Lamin C ΔN prompted us to examine the localization of Mlp84b (Muscle LIM protein 84b), which has the ability to bundle actin and contribute to muscle cell differentiation (Louis et al., 1997). Mlp84b is a member of the cysteine-rich protein (CRP) family, a group of cytoskeletal proteins that have conserved amino acid sequences that include one or more LIM domains, glycine-rich modules (Liebhaber et al., 1990; Louis et al., 1997; Weiskirchen and Guenter, 2003). Mlp84b is found in the nuclear and cytoplasmic compartments. Drosophila Mlp84b shares features with vertebrate MLPs including muscle-specific expression (Stronach et al., 1999). Third instar larvae expressing wild-type Lamin C in muscle stained with antibodies against Mlp84b showed proper localization resembling the sarcomeric striation pattern observed with Phalloidin (Fig. 3A). By contrast, larvae expressing Lamin C ΔN in muscle showed colocalization of Mlp84b and Phalloidin-positive actin fibers in muscle nuclei (Fig. 3A). These data suggest that, in the presence of mutant forms of Lamin C, Mlp84b might play a role in nuclear actin bundling.

These findings prompted us to examine the localization of Titin, a cytoskeletal protein that cooperates with Mlp84b to maintain muscle structural integrity (Clark et al., 2007). Titin binds lamins and has been proposed to contribute to nuclear organization during interphase (Zastrow et al., 2006). Drosophila Titin (D-Titin) is a large spring-like protein that shares sequence identity with

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Table 1. Tissue-specific expression of wild-type and mutant forms of Lamin C

<table>
<thead>
<tr>
<th>Driver</th>
<th>Stage</th>
<th>Tissue</th>
<th>WT</th>
<th>ΔN</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Act SC</td>
<td>Larval and adult</td>
<td>Ubiquitous</td>
<td>Viable</td>
<td>Lethal</td>
<td>Ito et al., 1997</td>
</tr>
<tr>
<td>C57</td>
<td>Larval</td>
<td>Muscle</td>
<td>Viable</td>
<td>Semi-lethal*</td>
<td>Koh et al., 1999</td>
</tr>
<tr>
<td>HOW</td>
<td>Larval</td>
<td>Larval muscle</td>
<td>Viable</td>
<td>Lethal</td>
<td>Brand et al., 1993</td>
</tr>
<tr>
<td>Mef2</td>
<td>Larval</td>
<td>Larval muscle</td>
<td>Viable</td>
<td>Lethal</td>
<td>Santiago-Martinez et al., 2006</td>
</tr>
<tr>
<td>CCAP</td>
<td>Larval</td>
<td>Brain</td>
<td>Viable</td>
<td>Viable</td>
<td>Park et al., 2003</td>
</tr>
<tr>
<td>C380</td>
<td>Larval</td>
<td>Type 1 motoneurons, epidermis, trachea</td>
<td>Viable</td>
<td>Viable</td>
<td>Koh et al., 1999</td>
</tr>
<tr>
<td>MHCF3</td>
<td>Adult</td>
<td>Muscle</td>
<td>Viable</td>
<td>Viable</td>
<td>Gift of W. Mattox laboratory</td>
</tr>
<tr>
<td>8171</td>
<td>Adult</td>
<td>Muscle</td>
<td>Viable</td>
<td>Viable</td>
<td>Seroude et al., 2002</td>
</tr>
<tr>
<td>8174</td>
<td>Adult</td>
<td>Muscle and brain</td>
<td>Viable</td>
<td>Viable</td>
<td>Seroude et al., 2002</td>
</tr>
<tr>
<td>8175</td>
<td>Adult</td>
<td>Muscle, cardi, testis</td>
<td>Viable</td>
<td>Viable</td>
<td>Seroude et al., 2002</td>
</tr>
<tr>
<td>8176</td>
<td>Adult</td>
<td>Brain, muscle, cardi,</td>
<td>Viable</td>
<td>Viable</td>
<td>Seroude et al., 2002</td>
</tr>
<tr>
<td>8182</td>
<td>Adult</td>
<td>Muscle</td>
<td>Viable</td>
<td>Viable</td>
<td>Seroude et al., 2002</td>
</tr>
<tr>
<td>8184</td>
<td>Adult</td>
<td>Muscle</td>
<td>Viable</td>
<td>Viable</td>
<td>Seroude et al., 2002</td>
</tr>
<tr>
<td>C929</td>
<td>Larval and adult</td>
<td>Brain, ring gland, salivary glands, fat body</td>
<td>Viable</td>
<td>Viable</td>
<td>Hewes et al., 2000</td>
</tr>
</tbody>
</table>

*Semi-lethal indicates that less than 90% of the anticipated class of progeny survive.
vertebrate titins, spans half the length of the sarcomere (Clark et al., 2007) and localizes to both the cytoplasm and nucleus in early Drosophila embryos (Machado and Andrew, 2000). Staining of third instar larvae expressing wild-type Lamin C in muscle with antibodies against D-Titin showed a typical striation pattern within the cytoplasm with no nuclear defects (Fig. 3B). Larvae expressing Lamin C ΔN in muscle showed filamentous structures surrounding the muscle nuclei without any detectable Titin staining of the nuclear Phalloidin rods (Fig. 3B). Thus, D-Titin localization is disrupted by the expression of Lamin C ΔN, but does not appear to play a direct role in nuclear actin filament formation.

Larval muscle-specific expression of Lamin C ΔN causes defects in leg morphogenesis

Expression of Lamin C ΔN using the GAL4 larval muscle-specific drivers frequently resulted in complete or semi-lethality, with at most 10% of the anticipated progeny surviving to adulthood (Table 1). The adult ‘escapers’ appeared normal upon expression of Lamin C ΔN with most drivers. However, in the case of the C57 driver, more than 50% of the escapers (n=326) showed defects in the third leg. These flies had a correct number of legs, all of which were properly segmented; however, the femur and tibia of the third leg were shorter and thinner in flies expressing Lamin C ΔN compared with flies expressing wild-type Lamin C. The basitarsus and the last tarsal segments were twisted and shorter in flies expressing Lamin C ΔN (Fig. 4A). These defects were apparent at the pupal stage, where the legs were observed to curl around the wing, rather than point to the distal end of the pupal case, as in wild type (Fig. 4B). Thus, Lamin C ΔN expression in the larval muscle hindered third leg morphogenesis.

The malformed adult leg phenotype suggested defects during leg disc elongation (Kiss et al., 1988; von Kalm et al., 1995). To determine when during morphogenesis this defect became apparent, we examined leg imaginal discs from wild-type and mutant prepupae. The shape of the leg imaginal disc cells can be examined by Phalloidin staining (Fortier et al., 2003). Discs from carefully staged pupae expressing wild-type Lamin C and Lamin C ΔN via the C57 driver were dissected and stained with Phalloidin to visualize filamentous actin. Discs were dissected six hours after pupariation formation (APF). Pupae that developed from larvae expressing Lamin C ΔN via the C57 driver possessed leg discs that
were misshaped and stunted in elongation compared with pupae that developed from larvae expressing wild-type Lamin C (Fig. 4C). In high magnification images of the control and Lamin C \( \Delta N \) mutant leg discs, differences in cell shape were observed (Fig. 4C).

**Leg defects are consistent with alterations in ecdysone signaling**

Leg imaginal discs are determined in embryogenesis and proliferate during larval development (Cohen et al., 1993). Leg elongation, morphogenesis and terminal differentiation depend on the steroid hormone ecdysone (20-hydroxyecdysone) (Fristrom et al., 1993; von Kalm et al., 1995). Ecdysone induces early response genes, which in turn regulate late genes directing downstream biological responses. The leg phenotype observed in pupae and adult escapers expressing Lamin C \( \Delta N \) via the C57 driver was remarkably similar to the phenotype observed in \( \beta Ftz-F1 \) mutant flies (Broadus et al., 1999). \( \beta FTZ-F1 \) acts as a competence factor for ecdysone induction of early genes \( E74, E93 \) and \( BR-C \) (Woodard et al., 1994). Loss-of-function alleles of \( \beta Ftz-F1 \) result in pupal lethality, with reduced expression of \( E74, E93 \) and \( BR-C \) in the late prepupal stage (Broadus et al., 1999). Given the lack of induction of \( \beta Ftz-F1 \) mRNA upon expression of Lamin C \( \Delta N \), we analyzed the expression levels of these downstream genes using RT-PCR. In flies expressing wild-type Lamin C, both \( E74 \) and \( E93 \) genes are maximally transcribed at mid-to-late prepupal stage (10 hours APF). At this stage, flies expressing Lamin C \( \Delta N \) showed suboptimal levels of induction of \( E74 \) and \( E93 \) mRNA (reduced by \( \sim 8 \)-fold). By contrast, \( BR-C \) mRNA was present at similar levels in wild-type Lamin C- and Lamin \( \Delta N \)-expressing flies (data not shown). A possible explanation for the lack of change in \( BR-C \) expression is that activation can occur via \( \beta FTZ-F1 \)-dependent and -independent pathways (Broadus et al., 1999; Karim and Thummel, 1992). Collectively, these findings show that larval muscle-specific expression of mutant Lamin C \( \Delta N \) results in lack of induction of \( \beta Ftz-F1 \) at the prepupal stage of development, which in turn fails to induce \( E74 \) and \( E93 \) early response genes.

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**Fig. 3. Cytoskeletal elements organization is perturbed in the muscles of larvae expressing Lamin C \( \Delta N \).** Staining of larval body wall muscle nuclei with (A) anti-Mlp84b or (B) anti-D-Titin antibodies (green) and Phalloidin (purple) in larvae expressing wild-type (wt) or Lamin C \( \Delta N \) (\( \Delta N \)) by the muscle-specific driver C57. Expression of Lamin C \( \Delta N \) causes accumulation of nuclear Mlp84b that colocalizes with nuclear actin fibers (yellow arrow, A) and a network of D-Titin around the nucleus (yellow arrows, B).
A role for Lamin C in gene regulation

Given that expression of Lamin C ΔN caused alterations in gene expression, we were interested in determining whether Lamin C could directly regulate gene expression. Laminas participate in genome organization and gene regulation. Lamina-genome interactions control gene expression during lineage commitment and terminal differentiation (Peric-Hupkes et al., 2010; Schneider and Grosschedl, 2007). The nuclear periphery is frequently lined with heterochromatin and is generally considered as a transcriptionally repressive environment. However, recent studies have suggested that in some cases active genes reside near the NE, perhaps in microdomains (Luo et al., 2009; Shimi et al., 2008). In the absence of known direct target genes for Lamin C, we developed a system that allowed us to determine the consequences of association of Lamin C with a reporter gene. This system involved the generation of ‘expressor’ stocks in which the hsp70 promoter was used to drive expression of a fusion protein comprising the DNA binding domain of the E. coli LacI repressor and full-length Lamin C. Using this system, we targeted Lamin C to a site upstream of two reporter genes (Fig. 6A). To determine whether effects were specific for A-type lamins, we generated a stock expressing the DNA-binding domain of the LacI repressor fused to Lamin Dm0. A stock expressing green fluorescent protein (GFP) fused to the DNA binding domain of the LacI repressor served as a negative control (Danzer and Wallrath, 2004). A stock expressing a fusion of LacI and Heterochromatin protein 1 (HP1) served as a positive control for gene silencing (Danzer and Wallrath, 2004). Each reporter stock possessed a P-element transposon containing a tagged version of hsp26 (hsp26-tag) and an hsp70-white reporter gene (Fig. 6A). Two heat-shock reporter genes were present at 1.9 and 3.7 kb distances downstream of 256 copies of lac operator repeats (Danzer and Wallrath, 2004; Robinett et al., 1996) (Fig. 6A). Reporter stocks differed only by the site of insertion of the lac-hsp26-hsp70 transposon as described in Table S2 in the supplementary material. Flies from the expressor stocks were crossed to flies from three separate reporter stocks. Progeny that resulted from crossing the expressor flies to the reporter flies were examined for nuclear position of the reporter genes and gene expression. Immunofluorescence staining using LacI antibodies was performed on polytene third instar salivary gland nuclei and diploid imaginal disc nuclei. The nuclear position of the reporter gene was scored as peripheral when intense staining at the reporter gene colocalized with anti-LacI staining at the nuclear periphery. Antibodies against LacI showed a focus of staining that localized to the nuclear periphery (Fig. 6A). The anti-LacI focus localized at the nuclear periphery in 96% and 98% of the cells expressing LacI-HP1 (D) and (E) E93 and (D) BR-C mRNA as ΔCT normalized to rp49 CT. The x-axis shows time relative to puparium formation (set at zero). Asterisk indicates $P<0.001$ as calculated by an unpaired Students’ t-test.

Fig. 5. Expression of ecdysone-responsive genes in staged pupae.
Quantitative RT-PCR analyses of mRNA from ecdysone-responsive genes. The y-axis indicates steady-state levels of (A) βFtz-F1, (B) E74, (C) E93 and (D) BR-C mRNA as ΔCT normalized to rp49 CT. The x-axis shows time relative to puparium formation (set at zero). Asterisk indicates $P<0.001$ as calculated by an unpaired Students’ t-test.

Fig. 4. Larval muscle-specific expression of Lamin C ΔN causes leg defects in adult legs and leg imaginal discs. (A,B) Third leg from adult flies (A) and pupae (B) expressing wild-type Lamin C (wt) or Lamin C ΔN (ΔN) by the C57 driver. Note the twisting of the leg in pupae and adult flies (red arrows) expressing Lamin C ΔN. (C) Leg discs from 6-hour pupae expressing wild-type (Lamin C wt) or N terminal deletion of Lamin C (Lamin C ΔN) by the C57 driver were dissected, fixed and stained with Phalloidin, which outlines the cell borders. Note the shortened leg length and unusual cell shape upon expression of Lamin C ΔN.

Whether expression of Lamin C ΔN caused altered gene expression in adult legs and leg imaginal discs, defects in adult legs and leg imaginal discs.
respectively (n=150; Fig. 6B). This single focus at the nuclear periphery was observed in both diploid and polytene nuclei. The remaining small percentage of the cells showed localization within the nuclear interior. Fluorescent in situ hybridization using a labeled oligonucleotide recognizing the lac repeats demonstrated colocalization of the anti-LacI signal and the reporter gene at the nuclear periphery (Fig. 6B). Thus, association of the reporter gene cassette with lamins, but not with GFP or HP1, caused the reporter genes to localize at the nuclear periphery.

To determine whether peripheral localization altered gene expression, transcript levels from the reporter genes were measured using RT-PCR analyses following a brief heat-shock treatment. RT-PCR analysis was performed using primers that measured using RT-PCR analyses following a brief heat-shock treatment. 

**DISCUSSION**

The function of the N-terminal domain of A-type lamins

The N-terminal head domain of A-type lamins in humans comprises ~33 amino acids that are not predicted to fold into a distinct structure (Herrmann and Aebi, 2004). Several studies have suggested that the head and tail domains are involved in lamin assembly (Herrmann and Foisner, 2003; Isobe et al., 2007; Sasse et al., 1998; Shumaker et al., 2005; Stuurman et al., 1998). We have determined the consequences of expressing the Drosophila A-type lamins lacking a head domain (Lamin C ΔN) in specific tissues. Most tissues were unaffected, whereas larval muscle was sensitive (Schulze et al., 2009). Similar to the cell culture studies (Izumi et al., 2000; Spann et al., 2002; Spann et al., 1997), the muscle cell nuclei were misshapen and exhibited disorganization of the nuclear pore complexes, Otefin, a SUN domain protein and the actin-tubulin cytoskeletal network, suggesting physical connections exist between the nucleus and cytoplasm. The fact that Lamin C ΔN caused these phenotypic consequences in the presence of endogenous wild-type Lamin C is consistent with a dominant-negative function, as has been suggested for several laminopathies (Burke and Stewart, 2006; Gotzmann and Foisner, 2006; Muchir et al., 2004; Walter et al., 2009). Expression of the headless A-type lamin caused disorganization of the nuclear pore complexes, Otefin, a SUN domain protein and the actin-tubulin cytoskeletal network, suggesting physical connections exist between the nucleus and cytoplasm. The fact that Lamin C ΔN caused these phenotypic consequences in the presence of endogenous wild-type Lamin C is consistent with a dominant-negative function, as has been suggested for several laminopathies (Burke and Stewart, 2006; Gotzmann and Foisner, 2006; Muchir et al., 2004; Walter et al., 2005).

**Actin in the nucleus**

Expression of Lamin C ΔN caused numerous defects in larval body wall muscles. One of the most striking defects was the presence of intranuclear Phalloidin staining fibers (Fig. 2). The formation of similar nuclear rods has been observed in stressed cells (Domazetovska et al., 2007; Fukui and Katsumaru, 1979; Ohta et al., 1989; Pendleton et al., 2003; Stuven et al., 2003; Welch and Suhan, 1985), in which Cofilin serves as a nuclear import factor.
(Ohta et al., 1989; Pendleton et al., 2003). In the Drosophila larval body wall muscle, we observed nuclear actin rod formation in the absence of Lamin C (Schulze et al., 2009) and upon expression of Lamin C ΔN. In Lamin C nulls, actin rods were apparent in muscle, but not diploid imaginal disc tissue, suggesting that formation is possibly linked with mechanical stress (Schulze et al., 2009). These findings are consistent with Lamin C playing a role in regulating the polymerization state of nuclear actin. Actin binds two regions in the Lamin A/C tail: residues 461-536 of the Ig-fold domain (55% amino acid identity with Drosophila Lamin C) and residues 563-646 (no significant identity with Drosophila Lamin C) (Zastrow et al., 2004). A growing body of evidence proposes that NE lamin-spanning complexes are structures capable of transmitting outside mechanical signals to the genome (Razafsky and Hodzic, 2009) through interactions made between nuclear actin and laminas, Emerin or nuclear myosin 1 (Gieni and Hendzel, 2009). The possibility that actin polymers are architectural partners of lamin meshwork is gaining support (Simon et al., 2010; Zastrow et al., 2004). In connection with human disease, actin fibers in both the cytoplasm and nucleus have been observed in several myopathies (Domazetovska et al., 2007; Hutchinson et al., 2006; Sparrow et al., 2003).

Connecting Lamin C to ecdysone signaling

Overt phenotypes caused by expression of Lamin C ΔN by the C57 larval muscle-specific driver, were death and the twisted leg phenotype of adult escapers (Table 1; Fig. 3). The remarkable similarity of the leg defect to that of ecdysone mutants (Broadus et al., 1999; Ward et al., 2003) suggested a connection between Lamin C and hormone-induced gene expression. In Drosophila melanogaster, fluctuations in ecdysone concentration coordinate major developmental processes such as morphogenesis during metamorphosis, gene expression and cell death. A high ecdysone concentration at the end of third instar larva initiates leg imaginal disc elongation and eversion. During leg elongation, ecdysone induces cell shape changes, which are thought to account for the elongation of basitarsal and tibial leg segments between 0 and 6 hours APF (Condic et al., 1991). Additionally, the contraction of larval body wall muscles is essential for leg elongation (Fortier et al., 2003). In directing leg formation and other developmental processes that occur during Drosophila metamorphosis, ecdysone acts to regulate a number of genes, including the orphan nuclear receptor βFtz-F1 (Broadus et al., 1999; Woodard et al., 1994). βFtz-F1 is expressed throughout the animal during the mid-prepupal period, reaching a peak at 8-12 hours APF (Condic et al., 1991). At 3 hours APF (Broadus et al., 1999; Ward et al., 2003) showed suboptimal induction in the mid-to-late prepupal stage (Fig. 5B,C), most likely owing to the failure of induction of βFtz-F1 at mid-prepupal stage upon expression of Lamin C ΔN (Fig. 5A). Lack of βFtz-F1 expression at this stage of development is known to limit muscle contractions necessary for leg extension (Fortier et al., 2003). Therefore, the leg phenotype resulting from reduced muscle function is probably due to two defects, disruption in the ecdysone signaling pathway and physical abnormalities in the muscle contractile apparatus.

The role of lamins in gene regulation

Lamins play a major role in genome organization and gene regulation (Schneider and Grosschedl, 2007). The nuclear periphery, which is generally considered a transcriptionally repressive environment, in some cases accommodates active genes (Luo et al., 2009; Shimi et al., 2008). Several studies have examined the interaction of lamins with DNA in living cells (Guelen et al., 2008; Pickersgill et al., 2006; Shevelyov et al., 2009). These studies used DamID, a process in which the E. coli DNA adenine methyl transferase (Dam) was fused to a B-type lamin and expressed in cultured cells. Methylated genomic DNA fragments are then presumed to be in contact with the NE. Results from such studies showed that lamin-associated domains (LADs) include large intergenic regions with a low gene density (Guelen et al., 2008).

The direct position of reporter loci near the NE in mammalian cells has been shown in three independent studies using the lacO-LacI system. Expression of LacI fused to either Lamin B1 (Kumarar and Spector, 2008), Emerin (Reddy et al., 2008) or the lamin-associated polypeptide 2β (LAP2β) (Finlan et al., 2008) were used to test the effects of tethering at the nuclear periphery on gene expression. Expression of LacI-Lamin B1 brought an inducible transgene array into juxtaposition with the nuclear lamina, but did not inhibit inducible gene activation (Kumarar and Spector, 2008). Repositioning of loci to the nuclear periphery using a LacI-Emerin fusion protein resulted in transcriptional repression of most, but not all, associated genes on the chromosome (Reddy et al., 2008). Similarly, peripheral tethering via a LAP2β-LacI fusion protein influenced the expression of some, but not all, endogenous genes near the lac operator repeats (Finlan et al., 2008). Collectively these data suggest that the repressive effects of the NE are gene-dependent. Perhaps weak promoters are silenced, whereas strong promoters retain the ability to recruit transcriptional machinery.

Our studies complement and expand upon these findings. For the first time, both A- and B-type lamins were used to tether reporter genes at the nuclear periphery. We used two reporter genes with similar promoters inserted at three different genomic sites. Targeting lamins caused the reporter gene to localize to the nuclear periphery (Fig. 6). The hsp26 promoter positioned 1.9 kb downstream of the lacO repeats was silenced by lamins at all genomic sites tested. This was not the case for the hsp70 promoter positioned 3.7 kb downstream of the repeats, which was silenced at 79D and 87C but not 4D5 (Fig. 6), despite that fact that nearly identical levels of LacI-Lamin C were associated at each genomic site (see Fig. S2 in the supplementary material). A plausible explanation for these results is that the transcriptional activity within the region 4D5 is relatively low during late third instar (the time at which RNA was collected) compared with the 79D and 87C genomic regions. The chromatin within of the region 4D5 is likely to be in a more closed configuration, as suggested by the low transcription level (http://Flybase.org) and low abundance epigenetic modifications found in transcriptionally active chromatin (http://modENCODE.org). By contrast, region 79D shows higher levels of transcriptional activity and higher abundance of H3K9 mono- and trimethylation. Similarly, region 87C contains heat-shock-induced transcripts, which are robustly transcribed upon the heat-shock induction required to produce the
Lacl-Lamin proteins. Our studies demonstrate that, in addition to promoter strength, genomic context dictates the ability of a gene to be transcribed at the nuclear periphery.

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Competing interests statement
The authors declare no competing financial interests.

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