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Tissue-Specific Defects Are Caused by Loss of the Drosophila MAN1 LEM Domain Protein

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ABSTRACT

The nuclear lamina represents a protein network required for nuclear structure and function. One family of lamina proteins is defined by an ~40-aa *L*AP2, *E*merin, and *M*AN1 (LEM) domain (LEM-D) that binds the nonspecific DNA-binding protein, barrier-to-autointegration factor (BAF). Through interactions with BAF, LEM-D proteins serve as a bridge between chromosomes and the nuclear envelope. Mutations in genes encoding LEM-D proteins cause human laminopathies that are associated with tissue-restricted pathologies. Drosophila has five genes that encode proteins with LEM homology. Using yeast two-hybrid analyses, we demonstrate that four encode proteins that bind Drosophila (d)BAF. In addition to dBAF, dMAN1 associates with lamins, the LEM-D protein Bocksbeutel, and the receptor-regulated Smads, demonstrating parallel protein interactions with vertebrate homologs. *P*-element mobilization was used to generate null *dMAN1* alleles. These mutants showed decreased viability, with surviving adults displaying male sterility, decreased female fertility, wing patterning and positioning defects, flightlessness, and locomotion difficulties that became more severe with age. Increased phospho-Smad staining in *dMAN1* mutant wing discs is consistent with a role in transforming growth factor (TGF)-β/bone morphogenic protein (BMP) signaling. The tissue-specific, age-enhanced *dMAN1* mutant phenotypes are reminiscent of human laminopathies, suggesting that studies in Drosophila will provide insights into lamina dysfunction associated with disease.

EUKARYOTIC cells are distinguished by the presence of a nucleus containing genomic DNA. The metazoan nucleus is enclosed by a double membrane that is underlined by the nuclear lamina, a network of proteins primarily composed of the nucleus-specific intermediate filament proteins, the A- and B-type lamins (Wilson *et al.* 2001; Goldman *et al.* 2004; Gruenbaum *et al.* 2005; Schirmer and Gerace 2005; Somech *et al.* 2005; Worman 2005). Lamins establish a structural platform for interactions with a wide variety of proteins. This extensive network is required for the maintenance of nuclear shape, chromosome organization, cell-cycle control, DNA replication, transcription, and RNA processing (Goldman *et al.* 2004; Gruenbaum *et al.* 2005; Dechat *et al.* 2008).

*L*AP2, *E*merin, and *M*AN1 (LEM) domain (LEM-D) proteins represent a family of nuclear lamina proteins that share an ~40-amino-acid domain, first identified in these three proteins (Lin *et al.* 2000; Wagner and Krohne 2007). This domain interacts with the small, conserved protein called barrier-to-autointegration factor (BAF) that binds double-stranded DNA and histones (Zheng *et al.* 2000; Cai *et al.* 2001; Laguri *et al.* 2001; Furukawa *et al.* 2003; Liu *et al.* 2003; Montes De Oca *et al.* 2005). Through interactions with BAF, LEM-D proteins connect interphase chromosomes with the nuclear lamina. Structural analyses of LEM-D proteins suggest that some of these proteins interact directly with chromatin. LAP2 contains a LEM-like domain that binds DNA (Cai *et al.* 2001), while MAN1 contains a MAN1-Src1p C-terminal (MSC) domain that forms a winged helix DNA-binding domain (Caputo *et al.* 2006). Together, these data provide evidence that LEM-D proteins are bridging proteins that contribute to nuclear organization.

LEM-D proteins are integral components of the extensive nuclear lamina network. These proteins associate directly with lamins (Holaska *et al.* 2003) and are required for nuclear envelope reformation during mitosis (Ashery-Padan *et al.* 1997a). LEM-D proteins interact with transcriptional repressors, such as germ cell-less and Bcl-2-associated transcription factor (Haraguchi *et al.* 2004; Mansharamani and Wilson 2005). In addition, interactions with the downstream transcriptional effectors of multiple signal transduction pathways have been observed, such as Smads [receptor-regulated (R)-Smads], retinoblastoma protein, and β-catenin (Markiewicz *et al.* 2002, 2006; Osada *et al.* 2003; Raju *et al.* 2003; Lin *et al.* 2005; Pan *et al.* 2005; Jiang *et al.* 2008). These latter observations suggest that LEM-D proteins have a role in the regulation of gene expression.

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Mutations in genes encoding LEM-D proteins cause human disease (Burke *et al.* 2001; Wilson *et al.* 2001; Lee and Wilson 2004; Gruenbaum *et al.* 2005; Somech *et al.* 2005; Worman 2005). Mutations in the gene encoding emerin (*EMD* or *STA*) are associated with *X*-linked familial atrial fibrillation (Ben Yaou *et al.* 2007; Karst *et al.* 2008), limb–girdle muscular dystrophy (Ura *et al.* 2007), and the recessive *X*-linked form of Emery–Dreifuss muscular dystrophy (EDMD) (Emery 2000). These diseases are typified by progressive skeletal muscle wasting and/or cardiac conductance defects. Interestingly, mutations in *LMNA*, the gene encoding A-type lamins, give rise to clinically similar diseases. These observations emphasize the link between the function of LEM-D proteins and lamins (Gruenbaum *et al.* 2005; Worman and Bonne 2007; Dechat *et al.* 2008). Heterozygous loss-of-function mutations in the human *MAN1* (*LEMD3*) gene cause a spectrum of disorders, including osteopoikilosis and the Buschke–Ollendorff syndrome, diseases characterized by increased bone density due to defective bone morphogenic protein (BMP) and transforming growth factor (TGF)-β signaling (Hellemans *et al.* 2004, 2006; Kawamura *et al.* 2005). Analysis of *LEMD3* mutant alleles suggests that disease phenotypes result from haploinsufficiency, as these alleles produce truncated proteins that lack the carboxyl-terminal protein recognition domain called the U2AF homology motif (UHM), a domain related to an RNA recognition motif (RRM) (Kielkopf *et al.* 2004). Loss of the UHM/RRM is proposed to increase TGF-β/BMP signaling due to the failure of MAN1 to interact with R-Smads (Osada *et al.* 2003; Raju *et al.* 2003; Lin *et al.* 2005; Pan *et al.* 2005). Disease phenotypes caused by *LMNA* mutations overlap with those caused by the loss of *MAN1* (Lee and Wilson 2004; Arimura *et al.* 2005), implying that the loss of one nuclear lamina component may alter the function of other proteins in the network.

Mechanisms responsible for phenotypes associated with LEM-D protein diseases remain poorly understood. These proteins are present in multiple tissues throughout development (Osada *et al.* 2003; Raju *et al.* 2003; Worman 2005), yet disease phenotypes are tissue restricted. To provide insights into LEM-D protein function, we have begun a molecular characterization of this family in *Drosophila melanogaster*. The genome contains five genes that encode proteins with LEM-D homology (Figure 1), including Drosophila *MAN1* (*dMAN1*) (Wagner *et al.* 2006), *bocksbeutel* (Wagner *et al.* 2004a), *otefin* (Padan *et al.* 1990; Goldberg *et al.* 1998), and the annotated genes *CG3748* and *dLEM3/CG8679* (Wagner *et al.* 2006). To date, molecular characterization has been restricted to dMAN1, Bocksbeutel, and Otefin. These proteins are produced through all stages of Drosophila development, with the levels of dMAN1 and Otefin most abundant in the embryo (Wagner *et al.* 2006). Immunolocalization studies demonstrate that dMAN1, Bocksbeutel, and Otefin are enriched at the nuclear envelope (Padan *et al.* 1990; Ashery-Padan *et al.* 1997a,b; Wagner *et al.* 2004a, 2006; Jiang *et al.* 2008). Drosophila provides an excellent model for studies of the developmental roles of the nuclear lamina proteins, as homologs of many vertebrate lamina proteins have been identified, including A- and B-type lamins (lamin C and lamin Dm_0, respectively) (Fisher *et al.* 1982; Lenz-Bohme *et al.* 1997; Osouda *et al.* 2005; Schulze *et al.* 2005), Drosophila (d)BAF (Furukawa *et al.* 2003), the lamin B receptor (Wagner *et al.* 2004b), and proteins in the linker of nucleoskeleton and cytoskeleton (LINC) complex (Starr and Fischer 2005; Stewart *et al.* 2007).

Studies described herein extend our understanding of the Drosophila LEM-D protein family, with an emphasis on dMAN1. Using yeast two-hybrid assays, we show that most of these genes (four of five) encode proteins that interact with dBAF, implying possible overlapping functions. Further, we find that dMAN1, Bocksbeutel, and Otefin directly interact with the A- and B-type lamins. Finally, we demonstrate that dMAN1 maintains protein associations found in vertebrate homologs, as the amino-terminal domain interacts with Bocksbeutel and the UHM/RRM interacts with R-Smads. To determine the role of dMAN1 in development, we used *P*-element mobilization to generate *dMAN1* null alleles. Interestingly, we find that *dMAN1* mutants display multiple tissue-specific defects without detectable changes in the localization and accumulation of other lamina components. Our data indicate that dMAN1 makes cell-type-specific contributions to nuclear lamina function and provide insights into how LEM-D proteins contribute to development.

MATERIALS AND METHODS

Yeast two-hybrid assay: The MATCHMAKER two-hybrid system 3 (Clontech, Palo Alto, CA) was employed to assess protein interactions. This system uses the *HIS3* and *ADE2* reporter genes to evaluate whether protein associations occur. Fusion genes encoding bait proteins were generated by cloning cDNAs in frame with sequences encoding the GAL4-binding domain present in pGBKT7, a vector that carries the *TRP1* gene as a selectable marker. Fusion genes encoding prey proteins were generated by cloning cDNAs in frame with sequences encoding the GAL4 activation domain in pGADT7, a vector that carries the *LEU2* gene as a selectable marker. Yeast cells transformed with both vectors were identified by growth in media lacking tryptophan and leucine (nonselective). Once obtained, transformed cells were streaked onto plates that lacked histidine and adenine, in addition to tryptophan and leucine (selective plates). An interaction was considered positive if incubation at 30° for 4 days produced thick and uniform growth on selective plates, with colonies ranging in color from a cloudy white to a dark pink. A negative interaction was indicated by lack of growth or only a few colonies on selective plates, with colonies showing a red color (supplemental Figure 1).

Drosophila stocks and culture conditions: Drosophila stocks were raised at 25° at 70% humidity on standard cornmeal/agar medium, with *p*-hydroxybenzoic acid methyl ester as a mold inhibitor. All crosses were maintained at 25°

and carried out in vials. The mutations and chromosomes used in this study are described in FlyBase (http://flybase.bio.indiana.edu/). Stocks were obtained from the Bloomington Stock Center, including *Df(2R)Chi[g230]* that removes cytological region 60A03-07–60B04-07.

Generation of *dMAN1* excision alleles and tests of viability: The starting *dMAN1 P*-element insertion line, *KG06361*, was generated in the Drosophila gene disruption project (Bellen *et al.* 2004). This line carries *SUPor-P* inserted into the 3′ end of the *dMAN1* gene at position +2416, located 24 nucleotides upstream of the translation stop codon. *SUPor-P* is marked with the *yellow*$^+$ (y^+) and *white*$^+$ (w^+) reporter genes (Roseman *et al.* 1995). Lines carrying an excision of *SUPor-P* were obtained using the chromosomal source of transposase, *P[ry*$^+$ *Δ2-3]* (99B) (Robertson *et al.* 1988). Females homozygous for the *KG06361* insert (y^1w^{67}; *KG06361*) were crossed to males that were y^+w^+/*Y; CyO/Sp; Δ2-3 Sb/TM6, Ubx*. Single red-eyed males (indicating the presence of *SUPor-P*) that carried the *CyO* (*Curly,* curly wings) and *Sb* (*Stubble*, short bristles, indicating *Δ2-3*) markers were mated to females that were y^+ w^{67}; *Sco/CyO;* +/+. Excisions (*KG**) were identified as white-eyed, non-*Sco* (*Scutoid*, loss of scutellar bristles), non-*Sb* flies (y^+ w^{67}/*Y; KG**/*CyO;* +/+). These flies were crossed to y^+ w^{67}; *Sco/CyO* flies and resulting non-*Sco*, *CyO* (y^+ w^{67}; *KG**/*CyO*) siblings were crossed to establish a stock.

Southern analysis was used to define the molecular structure of the *dMAN1* excision alleles. Genomic DNA was isolated from excision lines, digested with *Xmn*I (New England Biolabs, Beverly, MA), separated on an agarose gel, and transferred to a neutral nylon membrane (Nytran; Schleicher & Schuell Bioscience, Keene, NH). Hybridization was carried out with ^{32}P-labeled DNA probes corresponding to the *dMAN1* locus. After washing, filters were exposed to X-ray film to detect hybridization bands. On the basis of the resulting pattern, the endpoints of the deletion alleles were predicted and the precise endpoints were defined by PCR amplification and sequence analysis of the resulting DNA.

To determine effects of loss of *dMAN1* on viability, males and females carrying deletion alleles from the *KG* excisions over the *CyO* chromosome (*dMAN1*$^{\Delta}$/*CyO*) were mated. The resulting progeny were screened for the presence or absence of curly wings (*CyO*). These analyses demonstrated that fewer homozygous *dMAN1* mutants were obtained than expected on the basis of the number of *CyO* flies. To discern when in development *dMAN1* mutants died, y^1w^{67c23}/*Y; dMAN1*$^{\Delta 81}$/y^+ *CyO* males were crossed with $y^1w^{67c23}/y^1w^{67c23}$; *dMAN1*$^{\Delta 81}$/$y^+$ *CyO* females and resulting embryos were collected (~250). After 24 hr, hatched larvae were examined for pigmentation of the larval mouthparts to identify heterozygous (dark pigmentation) and homozygous *dMAN1*$^{\Delta 81}$ mutant (light pigmentation) larvae. Each group was counted and put into a separate vial, and resulting numbers of pupae and adults were determined. From these data, a survival percentage was calculated, on the basis of the number of embryos in the original collection. We assumed that 100% of heterozygous embryos hatched, allowing calculation of an estimated number of homozygous *dMAN1*$^{\Delta 81}$ mutants, assuming Mendelian ratios. Parallel studies were done on collections of y^1w^{67c23} embryos, as controls. To examine whether loss of maternal dMAN1 protein affected offspring viability, we crossed y^1w^{67c23}/*Y; dMAN1*$^{\Delta 81}$/y^+ *CyO* males with $y^1w^{67c23}/y^1w^{67c23}$; *dMAN1*$^{\Delta 81}$/*dMAN1*$^{\Delta 81}$ females, collected embryos, and followed a similar analysis as described above. For each set of analyses, three independent experiments were completed.

Generation of the *dMAN1* rescue construct: The *dMAN1* genomic rescue construct *P[dMAN1-gen]* was made by PCR amplification of y^1w^{67c23} genomic DNA, using the Expand High Fidelity system (Roche Diagnostics, Indianapolis). The forward primer used was 5′-GCCTCCACCGATAGTTTTGCCATC-3′ and the reverse primer used was 5′-CGCTCTGTCGTCCA ACTCCCTAG-3′. The resulting 2.9-kb fragment extended from −306 to +2605 relative to the *dMAN1* transcription start site and was cloned into the pCR2.1-TOPO vector (Invitrogen, San Diego). This fragment lacked the *Chip* and *CG13567* genes. Sequence analysis of the amplified *dMAN1* genomic region showed 11 silent substitutions when compared with published sequences (http://flybase.bio.indiana.edu/). The *dMAN1* genomic region was cloned into the *P*-element germ-line transformation vector pCaSpeR-3 and transgenic flies were produced. Southern analysis was performed on each transgenic line to determine the integrity and copy number of the transgene.

Generation of dMAN1 antibody: Polyclonal sheep anti-dMAN1 antibodies were generated against the carboxyl-terminal domain (CTD) of dMAN1 that included amino acids 425–650, using the following primers: 5′-CATATGAAGCA GAAGGAAGCCCTGTTCCG-3′ (forward) and 5′- CTCGAGT GAGTGAGTGTTGGCTGCCTCGTTG-3′ (reverse). The amplified fragment was cloned into the pCR2.1-TOPO vector, sequenced, digested with *Nde*I and *Xho*I, and cloned into the expression vector pET21-a (Novagen), forming pET-dMAN1-CTD. This plasmid was transformed into BL21-DE3 cells (Invitrogen) and expression of the fusion protein was obtained by incubation with IPTG overnight at 18°. The resulting dMAN1-CTD-His fusion protein was purified over a Ni^{2+} column (QIAGEN, Valencia, CA) and used to immunize sheep (Elmira Biologicals, Iowa City, IA). The resulting antibody was affinity purified using amino acids 425–539 of dMAN1 (Actigel, Sterogene), corresponding to sequences that encompass the dMAN1 MSC domain, but not the UHM/RRM.

Western analysis and immunohistological analyses: For Western analysis of fly extracts, proteins were extracted from 10 adults, separated on an 8% polyacrylamide gel, transferred to a nitrocellulose membrane, and incubated overnight at 4°. Blots were incubated with secondary antibodies HRP-conjugated donkey anti-sheep IgG [Sigma (St. Louis) no. A3415] and HRP-conjugated rabbit anti-mouse IgG (Sigma no. A9044) for 2 hr and detected using the SuperSignal West Pico chemiluminescent substrate [Pierce (Rockford, IL) no. 34080]. To control for amounts of protein loaded, blots were incubated with the mouse anti-α-tubulin IgG primary antibody (Sigma no. T5168) and detected with the HRP-conjugated rabbit anti-mouse IgG secondary antibody (Sigma no. A9044).

For immunohistological analyses, salivary glands and wing imaginal discs were dissected from third instar larvae and placed in phosphate-buffered saline solution (PBS). Dissections were completed in <1 hr. Tissues isolated from three to five larvae were fixed in 2–4% paraformaldehyde for 15–20 min, followed by three 5-min washes in PBS^{2+} (130 mM NaCl, 7 mM Na_2HPO_4, 3 mM NaH_2PO_4, 10 mM EGTA, 0.1% Triton-X-100). Prior to overnight incubation with primary antibodies at 4°, tissues were blocked in PBS^{2+} + 0.1% BSA for 1 hr or permeabilized with 0.3% Triton X-100 in PBS for staining of nuclear lamina components and phospho-Mad, respectively. After three 5-min washes with PBS^{2+}, tissues were incubated for 2 hr in the dark at room temperature with the following secondary antibodies: Alexa Fluor 488-conjugated goat anti-mouse IgG (Invitrogen, Molecular Probes, no. A-11001), Texas Red-conjugated donkey anti-guinea pig IgG [Jackson ImmunoResearch Labs (West Grove, PA) no. 706-075-148], Alexa Fluor 546-conjugated donkey anti-sheep IgG (Invitrogen, Molecular Probes, no. A-21098), or Alexa Fluor 568-conjugated goat anti-rabbit IgG (Invitrogen, Molecular Probes, no. A-11011). Following the secondary antibody treatment, tissues were washed three times for 10 min in PBS^{2+} and mounted in Vectashield H-1000 (Vector Laboratories, Burlingame, CA). Slides were examined using a Nikon E600

microscope (60× objective) with fluorescent capabilities. Images were obtained and processed with the Bio-Rad (Hercules, CA) MRC 1024 confocal laser scanning imaging system.

For analysis of the ovarian phenotype associated with *dMAN1* mutants, mated wild-type (y^1w^{67c23}) and $dMAN1^{\Delta 81}$ females were raised on food supplemented with yeast granules for 3–4 days prior to ovary dissection. Ovaries were dissected in PBS, fixed with heptane-saturated 3% formaldehyde in 0.3% Triton X-100 in PBS (PBT), washed with PBT, and stained with DAPI (0.1 μg/ml in PBT). Stained ovaries were mounted in a drop of Vectashield H-1000 (Vector Laboratories) and slides were examined using an Olympus BX-51 light microscope with fluorescent capabilities. Images were processed using the Olympus DP imaging software.

Primary antibodies used in these studies include sheep anti-dMAN1, mouse anti-lamin C (University of Iowa Hybridoma Core Facility, LC28.26), mouse anti-lamin Dm_0 (University of Iowa Hybridoma Core Facility, ADL84.12), mouse anti-Otefin (a generous gift from Y. Gruenbaum), mouse anti-HP1 (L. L. Wallrath), guinea pig anti-Bocksbeutel (a generous gift from G. Krohne), mouse anti-NPC (Covance, MMS-120P), and rabbit anti-phospho-Smad1/5 (Ser463/465; Cell Signaling Technology, 41D10).

Climbing assays: Climbing assays were performed as described previously (Sun and Tower 1999), with minor modifications. Briefly, five males and five females for each genotype were placed in a 30-cm-long by 1.5-cm-wide graduated plastic cylinder. After placement, the flies were equilibrated for several minutes, gently tapped to the bottom, and allowed to climb up the sides. The number of flies that crossed the 30-cm mark in a 1-min time period was recorded. This procedure was repeated five times, allowing 1-min rest intervals. The assay was replicated 10 times with 10 different groups of flies ($n = 100$). The average of these replicates was plotted as the percentage of flies that climb 30 cm in 1 min at 2 and 10 days of age.

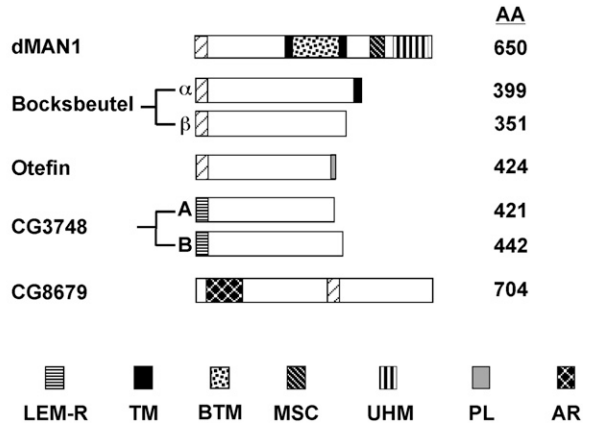

Figure 1.—Structure of the Drosophila LEM domain proteins. The Drosophila genome has five genes that encode proteins with LEM homology: *dMAN1*, *bocksbeutel*, *otefin*, *CG3748*, and *CG8679/dLEM3*, of which *bocksbeutel* and *CG3748* each encode two isoforms. dMAN1, Bocksbeutel, Otefin, and dLEM3 possess LEM domains, while CG3748 possesses a LEM-related (LEM-R) domain. Outside of the LEM homology domain, these proteins have unique structures including transmembrane domains (TM), a between the transmembranes (BTM) domain, the MAN1-Src1-p C-terminal (MSC) domain, the U2AF homology motif (UHM), a peripheral localization signal (PL), and ankyrin repeats (AR). The size, in amino acids (AA), is shown.

RESULTS

Interaction partners of the Drosophila LEM-D proteins: The Drosophila genome contains five genes encoding proteins that have homology with the LEM-D (Figure 1). We tested whether these LEM homology proteins bind dBAF, a defining property of vertebrate LEM-D proteins. DNA sequences corresponding to each LEM homology domain were isolated by PCR amplification and cloned into the yeast two-hybrid vector pGBKT7 to generate bait constructs. *dBAF* cDNA sequences were cloned into pGADT7 to generate the prey vector. Transformation with individual bait or prey clones demonstrated that none of these proteins activated transcription alone (data not shown).

Interactions between the LEM homology domain proteins and dBAF were tested by cotransformation of yeast with bait and prey vectors, plating cells onto nonselective media and restreaking resulting colonies onto selective media. We found that in all but one case, the doubly transformed yeast cells grew on selective media, indicating a positive protein interaction (supplemental Figure 1). The exception was cells carrying the *CG3748* expression vector, suggesting that this protein does not have a LEM-D. Western analyses indicated the level of accumulation of the protein containing the CG3748 domain was not responsible for the absence of interaction (supplemental Figure 1). Interestingly, among the Drosophila proteins, CG3748 has the lowest conservation of the LEM homology region with human (h)MAN1, with 25% identity and 44% similarity (data not shown). For comparison, the dMAN1 LEM-D shows 37% identity and 61% similarity to hMAN1 LEM-D (supplemental Table 1). The homology of the CG3748 domain to the LAP2 LEM-like DNA-binding domain is also low (15% identity, 40% similar) (Cai *et al.* 2001; Laguri *et al.* 2001). For these reasons, we refer to the region of CG3748 as a LEM-related domain. We conclude that the Drosophila genome contains four genes that encode *bona fide* LEM-D proteins (Figure 1).

Three Drosophila LEM-D proteins require lamin Dm_0 for localization to the nuclear envelope (Wagner *et al.* 2006). To test whether these effects reflect direct interactions between LEM-D proteins and lamins, we cloned the cDNA sequences for the LEM-D proteins and the A- and B-type lamins into yeast two-hybrid vectors to test for association. We found that cotransformation of yeast with the dMAN1, Bocksbeutel, or Otefin expression vector and one of the two lamin expression vectors (lamin Dm_0 or lamin C) resulted in growth on selective media (data not shown). In contrast, yeast carrying the lamin expression vectors and expression vectors for CG3748ΔLEM or CG8679 failed to grow, despite detection of these proteins by Western analyses (data not shown). These experiments suggest that lamin Dm_0

TABLE 1

Yeast two-hybrid interactions for dMAN1 domains

dMAN1 domain	dBAF (AD)	Bocks[a] (BD)	Lamin Dm_0 (BD)	Lamin C (BD)	Mad (BD)	dSmad2 (BD)	Med (BD)	Dad (AD)
LEM	+	−	−	−	−	−	−	−
NTDΔLEM[b]	−	+	+	+	−	−	−	−
BTM	−	−	−	−	−	−	−	+
MSC[c]	−	−	−	−	−	−	−	−
UHM	−	−	−	−	+	+	−	−

BD, GAL4 binding domain; AD, GAL4 activation domain; +, positive interaction; −, negative interaction.
[a] Bocks, Bocksbeutel α- and β-isoforms.
[b] NTDΔLEM contains amino acids 61–228.
[c] MSC contains amino acids 425–539.

targeting of dMAN1, Bocksbeutel, and Otefin to the nuclear envelope involves direct interactions.

dMAN1 contains structurally and functionally conserved domains: dMAN1 displays features of both hMAN1 and LEM2. The amino terminus of dMAN1 is shorter than that of hMAN1 and similar in size to that of LEM2. The carboxyl terminus of dMAN1 contains a UHM/RRM domain, a domain present in hMAN1 but absent in LEM2 (Mansharamani and Wilson 2005). We compared the amino acid sequences of dMAN1 with MAN1 from several invertebrate and vertebrate species, to gain insights into the degree of conservation of the structural domains. These analyses demonstrated a high degree of amino acid identity and similarity in the LEM, MSC, and UHM domains (supplemental Table 1). In addition, we found that conservation exists in the 140-amino-acid region located between the two transmembrane (BTM) domains (39% similarity between dMAN1 and LEM2, as compared to 43% similarity for the LEM-Ds). As the BTM domain resides in the perinuclear space, these observations raise the intriguing possibility that dMAN1 functions might not be restricted to the nucleus.

We tested the protein-interaction properties of individual domains of dMAN1 to determine whether these motifs possess conserved function. Five distinct structural domains were isolated by PCR amplification of the *dMAN1* cDNA and these fragments were cloned into yeast two-hybrid vectors. In all cases, the resulting bait and prey fusion proteins stably accumulated in yeast and lacked intrinsic transcriptional activation potential (data not shown). First, individual domains of dMAN1 were tested for interaction with nuclear lamina proteins (Table 1). We found that the amino-terminal domain located between the LEM and first transmembrane domain (NTDΔLEM) associated with Bocksbeutel, lamin Dm_0, and lamin C, mirroring domain interactions seen with hMAN1 (Mansharamani and Wilson 2005). Our studies showed that dBAF interactions were limited to the LEM-D, unlike BAF association with hMAN1 that occurs with both the LEM-D and amino acids in the carboxyl terminus (Mansharamani and Wilson 2005). Second, we tested whether dMAN1 domains associated with Drosophila Smad proteins, transcription factors that are the downstream effectors of the Decapentaplegic (Dpp) signaling pathway that is analogous to the vertebrate TGF-β/BMP pathway. The Drosophila Smad family contains four members, including Mothers against Dpp (Mad), corresponding to the BMP pathway-specific R-Smad; dSmad2, corresponding to the TGF-β/activin pathway-specific R-Smad; Medea, corresponding to the common mediator Smad (co-Smad); and Daughters against Dpp (Dad), corresponding to a Smad antagonist (anti-Smad) (Raftery and Sutherland 1999). We found that the UHM/RRM domain associated with Mad and dSmad2, but not Medea or Dad, demonstrating the same specificity for R-Smads as seen with vertebrate MAN1 (Osada *et al.* 2003; Raju *et al.* 2003; Lin *et al.* 2005; Pan *et al.* 2005). Surprisingly, we found that the BTM domain interacted with Dad (Table 1), providing a possible explanation for the observed conservation. Taken together, our data imply that dMAN1 is an integral part of the Drosophila nuclear lamina network, displaying interaction partners conserved with vertebrate homologs.

Loss of dMAN1 reduces viability: The *dMAN1* gene maps to cytological position 60B5 on the right arm of chromosome 2. In this gene-dense region, *dMAN1* is located 270 bp downstream of the divergently transcribed gene *Chip* and 74 bp upstream of the annotated gene *CG13567* (Figure 2A). To generate mutant *dMAN1* alleles, we mobilized the *P* transposon in the *KG06361* line that is located at +2416 relative to the *dMAN1* transcription start site. Flies homozygous for the *KG06361* insertion are viable and fertile and show no visible mutant phenotypes. These flies were crossed to a source of *P* transposase and 108 excision lines were generated. Southern analysis was used to identify whether structural changes occurred in the *dMAN1* locus. We determined that several excision events resulted in deletion of sequences in the *dMAN1* locus (Figure 2A). Importantly, we found one line where the deletion was limited to the *dMAN1* gene, starting at position +92 relative to the *dMAN1* transcription start site and extending to +2416, with 240 bp of the *SUPor-P* element remaining at the initial insertion site. We call this line

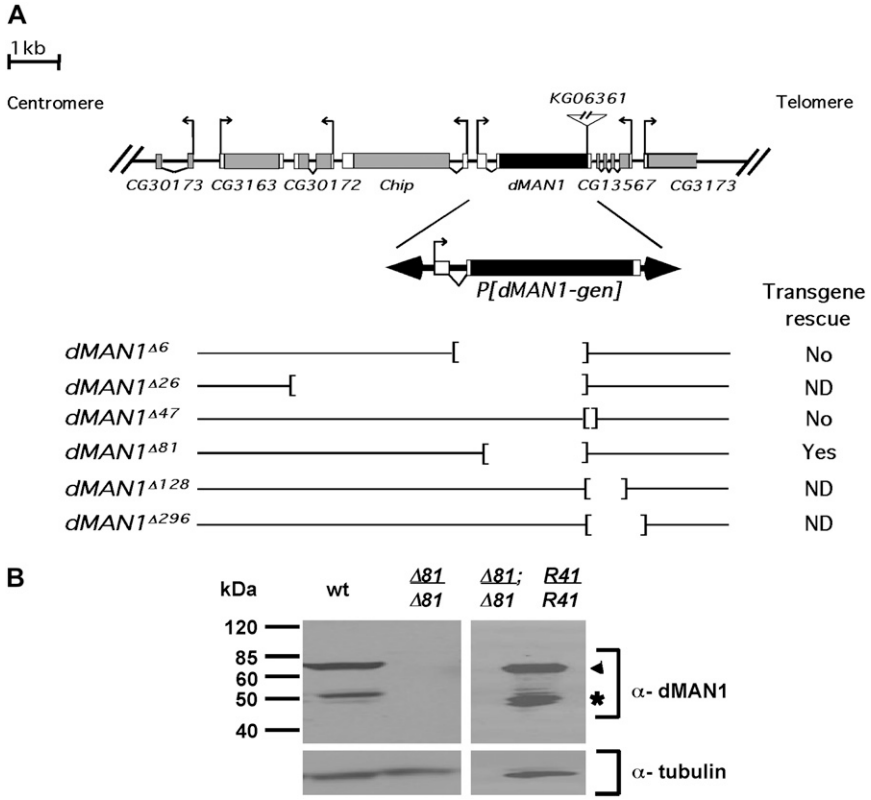

FIGURE 2.—Generation and analysis of *dMAN1* deletion alleles. (A) Structures of the *dMAN1* genomic region and genomic rescue construct. *dMAN1* is located downstream of the essential gene *Chip* and upstream of the uncharacterized gene *CG13567*. The location of the *P* element in *KG06361* (triangle at top) is shown. The *dMAN1* genomic rescue transposon, *P[dMAN1-gen]* is shown below the genomic region (not drawn to scale) and does not include transcribed regions of adjacent genes. Shown are the structures of deletion alleles, generated by *P*-element excision, with limits of the deleted sequences represented by brackets. With respect to the start site of transcription of *dMAN1*, Δ*6* extends from −551 to +2416, Δ*26* extends from −3601 to +2416, Δ*47* extends from +2416 to +2626, Δ*81* extends from +92 to +2416, Δ*128* extends from +2416 to +3488, and Δ*296* extends from +2416 to +3932. The ability of *P[dMAN1-gen]* to rescue the mutant phenotypes associated with these deletions is indicated to the right. (B) Western analysis of dMAN1 production. Adult protein extracts representing one fly equivalent from wild type (wt), $dMAN1^{\Delta 81}$ (Δ*81*/Δ*81*) homozygotes, and $dMAN1^{\Delta 81}$ homozygotes carrying the *P[dMAN1-gen]* rescue construct, R41 (Δ*81*/Δ*81; R41*/*R41*) were isolated and run on an SDS–polyacrylamide gel. Westerns were incubated with antibodies against dMAN1. The positions of the full-length protein (arrowhead) and degradation products (asterisk) are shown. α-Tubulin was used as a loading control.

$dMAN1^{\Delta 81}$. Western analysis, using an antibody generated against the dMAN1 carboxyl-terminal MSC domain, failed to detect a protein in extracts from $dMAN1^{\Delta 81}$ homozygotes (Figure 2B), implying that $dMAN1^{\Delta 81}$ represents a null allele.

Complementation tests were conducted between $dMAN1^{\Delta 81}$ and other excision alleles in the *60B* region (Table 2). These crosses included a self cross of $dMAN1^{\Delta 81}/CyO$, a cross with the lethal excision line, $dMAN1^{\Delta 6}/CyO$, and a cross with an independently generated deficiency line, *Df(2R)Chi[g230]*/*CyO*, that carries a deletion that includes cytological position 60B. The deficiency is an important genetic tool, as it includes a deletion of the *dMAN1* gene in an unrelated genetic background. Progeny were collected, and the numbers of non-*CyO* and *CyO* flies were determined. We found that fewer $dMAN1^{\Delta 81}$ homozygotes eclosed than predicted on the basis of the number of $dMAN1^{\Delta 81}/CyO$ siblings, representing 57% of the expected number (Table 2). Crosses with the other deletion alleles also produced reduced numbers of $dMAN1^{\Delta 81}$ homozygotes, suggesting that loss of *dMAN1* reduces viability. Further, the absence of complementation between $dMAN1^{\Delta 81}$ and *Df(2R)Chi[g230]* demonstrates linkage of the reduced viability to cytological position 60B.

P-element mobilization events can be complex and involve multiple loci. To verify that the reduced viability associated with $dMAN1^{\Delta 81}$ homozygotes was due to the absence of dMAN1, we generated transgenic lines that carried a genomic rescue construct, *P[dMAN1-gen]*. This

TABLE 2

Progeny numbers obtained from complementation analyses with *dMAN1* alleles

	$\frac{KG06361}{CyO}$		$\frac{\Delta 6}{CyO}$		$\frac{\Delta 81}{CyO}$		$\frac{Df(2R)}{CyO}$	
	KG (%)[a]	*CyO*	Δ*6* (%)	*CyO*	Δ*81* (%)	*CyO*	*Df* (%)	*CyO*
KG06361	96 (94)	204	31 (128)	49	38 (81)	94	ND	ND
Δ*6*	38 (112)	68	0 (0)	506	77 (62)	247	0 (0)	171
Δ*81*	38 (103)	73	45 (63)	141	100 (57)	348	2 (5)	73
Df(2R)[b]	ND	ND	0 (0)	133	9 (7)	256	0 (0)	405

[a] Percentage of expected progeny, as determined from the number of *CyO* progeny.
[b] *Df(2R)* represents *Df(2R)Chi[g230]* that removes 64 genes, including *dMAN1*.

transposon carries a fragment of the 60B region that contained only sequences of the *dMAN1* gene (Figure 2). Once transgenic lines were established, flies from three independent insertions were crossed into a $dMAN1^{\Delta 81}$ or a $dMAN1^{\Delta 6}/CyO$ mutant background to test for complementation. As expected, *P[dMAN1-gen]* rescued the decreased viability of the $dMAN1^{\Delta 81}$ homozygotes. We obtained $dMAN1^{\Delta 81/\Delta 81}$; *P[dMAN1-gen]* progeny at 92–115% of the expected class, while $dMAN1^{\Delta 81/\Delta 81}$ siblings were obtained at 54–56% of the expected class (data not shown). However, the lethality associated with $dMAN1^{\Delta 6}$ was not rescued, as *P[dMAN1-gen]* lacks the essential gene *Chip*. Western analysis demonstrated that production of dMAN1 protein was restored in $dMAN1^{\Delta 81}$ homozygotes that carried *P[dMAN1-gen]* (Figure 2B). These results confirm that the reduced viability associated with flies carrying $dMAN1^{\Delta 81}$ is specifically caused by loss of *dMAN1*.

dMAN1 is required during two developmental periods: We crossed $y^1\ w^{67}$; $dMAN1^{\Delta 81}/y^+\ CyO$ females and males to investigate the consequences of the loss of zygotically produced dMAN1 on development. Embryos from this cross were collected and hatched larvae were genotyped by examination of cuticle pigmentation. Heterozygotes had dark mouth parts ($yellow^+$) and homozygotes had light mouth parts ($yellow^-$). Larvae of each genotype were placed into separate vials and allowed to develop. The number of resulting pupae and adults was determined and the percentage of survival of each genotype was calculated, on the basis of the actual or predicted number of embryos collected in each class (see MATERIALS AND METHODS). These studies revealed that the majority of *dMAN1* homozygotes were lost during the pupal-to-adult transition (Figure 3A). As this time period involves imaginal disc differentiation, these data suggest a requirement for dMAN1 in these processes. A similar strategy was used to examine effects of the absence of both maternally and zygotically supplied dMAN1 on development. For these experiments, we crossed $y^1\ w^{67}$; $dMAN1^{\Delta 81}$ homozygous females to $y^1\ w^{67}$; $dMAN1^{\Delta 81}/y^+\ CyO$ males and followed the strategy outlined above. In this case, $dMAN1^{\Delta 81}$ homozygotes hatched at only ~8% of the predicted number (Figure 3B). Surprisingly, a few of these larvae survived to adulthood, but died within a day of eclosion. On the basis of these data, we conclude that dMAN1 plays an important role during embryogenesis and metamorphosis.

Loss of dMAN1 is not essential for nuclear lamina formation: We investigated whether the absence of dMAN1 affected nuclear structure and organization. As dMAN1 directly interacts with several lamina components (Table 1), we reasoned that loss of this protein may alter the stability or assembly of proteins into the nuclear lamina. Western analyses of proteins extracted from adults demonstrated that loss of dMAN1 had no effect on accumulation of lamin Dm_0, lamin C, Otefin, or HP1

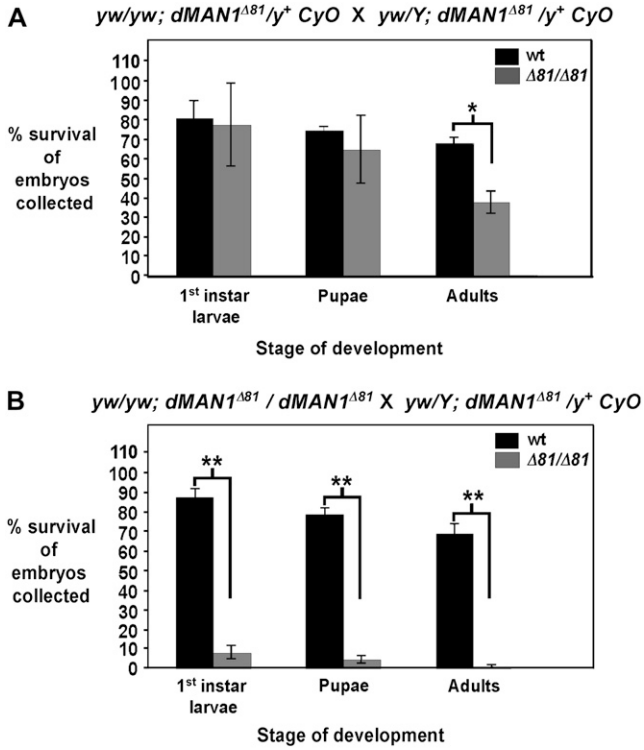

FIGURE 3.—Effects of loss of maternal and zygotic dMAN1 on development. (A and B) Embryos were collected from crosses between *yw/Y; dMAN1*$^{\Delta 81}$*/y+ CyO* males and *yw/yw; dMAN1*$^{\Delta 81}$*/y+ CyO* females (A) or *yw/yw; dMAN1*$^{\Delta 81}$ homozygous females (B). Wild-type (wt, y^1w^{67}) embryos served as a control. The percentage of embryos that survived to first instar larval, pupal, and adult stages was calculated. Shown is the average percentage of survival of wild-type (wt) (solid bars) and $dMAN1^{\Delta 81}$ homozygous ($\Delta 81/\Delta 81$; shaded bars) embryos from three experiments. Error bars represent the mean plus or minus standard deviations. (A) $^*P < 0.01$ and (B) $^{**}P < 0.005$, calculated using Student's *t*-test.

(Figure 4A). Nuclear localization of lamina components in $dMAN1^{\Delta 81}$ mutants was studied in tissues isolated from third instar larvae, as the maternally contributed protein is degraded by this time period (data not shown). We studied salivary gland nuclei because the large size facilitates visualization of protein distribution and wing disc nuclei that represent a diploid tissue affected in the mutant. These studies showed that neither nuclear morphology nor the subcellular distribution of any of the tested lamina components was altered in *dMAN1* mutants (Figure 4B). We found that the lamins, nuclear pore complexes, Otefin, and Bocksbeutel associate with the nuclear lamina in the absence of dMAN1. This latter finding is particularly interesting, as dMAN1 directly interacts with Bocksbeutel (Table 1). These findings agree with previous studies in Drosophila Kc167 cells (WAGNER *et al.* 2006), implying that LEM-D proteins and other lamina components do not require dMAN1 for nuclear envelope localization.

LEM-D proteins are proposed to contribute to nuclear organization through interactions with BAF that bridge the nuclear envelope and chromatin. To test

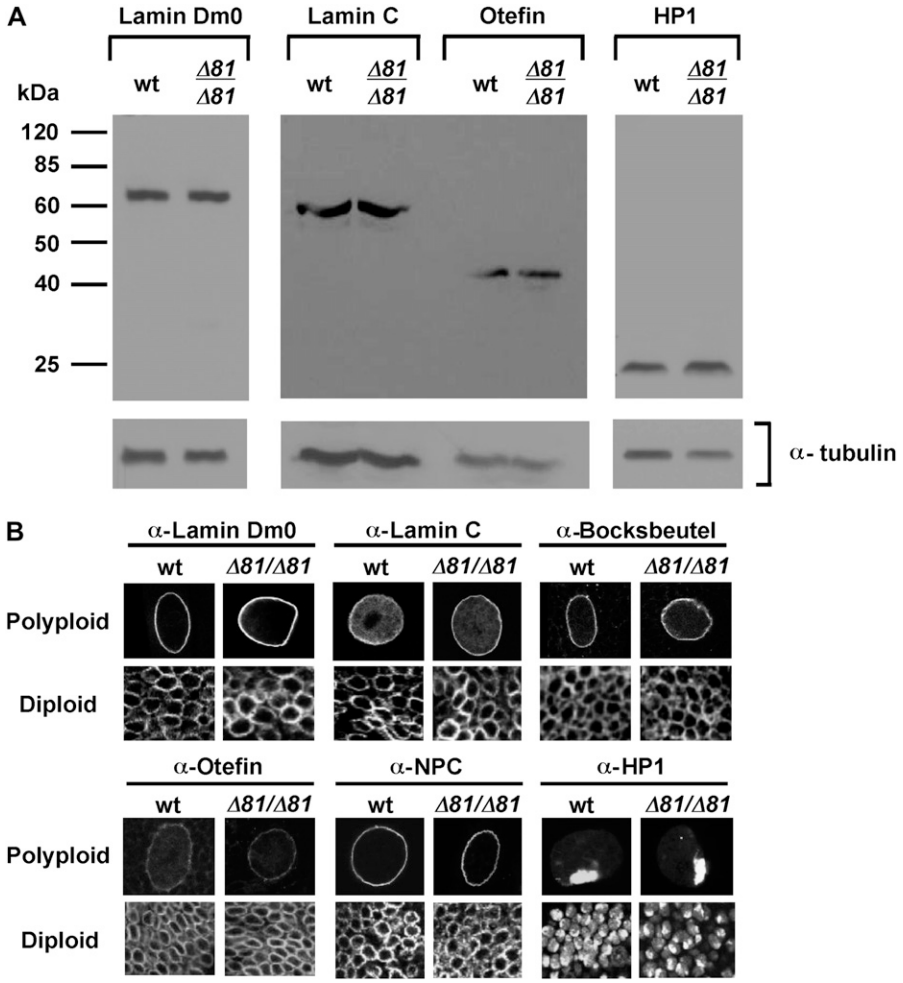

FIGURE 4.—Western and immunocytochemical analyses of nuclear lamina structure in *dMAN1* mutant tissues. (A) Western analysis of the level of accumulation of nuclear lamina components in *dMAN1* mutants. Proteins were isolated from wild-type (wt, Canton S) and $dMAN1^{\Delta 81}$ homozygous ($\Delta 81/\Delta 81$) adults. One fly equivalent per lane was loaded on an SDS–polyacrylamide gel. Westerns were analyzed with antibodies against lamin Dm_0, lamin C, Otefin, and HP1. α-Tubulin represents the loading control. (B) Immunocytochemical analysis of nuclear lamina components in polyploid (larval salivary glands) or diploid (wing imaginal disc) tissues of *dMAN1* mutants. Tissues were isolated from wt and $\Delta 81/\Delta 81$ third instar larvae and stained with antibodies to nuclear lamina components (lamin Dm_0, lamin C, Bocksbeutel, and Otefin), nuclear pore complex (NPC) proteins containing FG repeats, and heterochromatin protein 1 (HP1).

whether chromosome organization was disrupted in *dMAN1* mutant flies, we examined the localization of HP1, a structural component of heterochromatin that serves as a marker for heterochromatin distribution. In both wild-type and $dMAN1^{\Delta 81}$ mutants, HP1 localized to the nuclear periphery in a single large domain, corresponding to the chromocenter that represents a fusion of centromeres (Figure 4B). These observations indicate that dMAN1 is not essential for heterochromatin positioning.

Loss of *dMAN1* causes tissue-specific phenotypes: Although nuclear organization was not detectably altered in $dMAN1^{\Delta 81}$ homozygotes, adult flies displayed several visible phenotypes. These defects are described below. In all cases, mutant phenotypes were completely rescued by the *P[dMAN1-gen]* transposon.

dMAN1 mutant flies showed wing-patterning defects, wherein the stereotypical organization of longitudinal veins and crossveins was disrupted (Figure 5A). Commonly, the wings of $dMAN1^{\Delta 81}$ homozygotes showed thickening of all longitudinal veins, a variable number of anterior crossveins (1–4), branching of the posterior crossvein, and folds in the blade. These phenotypes are reminiscent of the mutant phenotypes associated with ectopic Dpp signaling, wherein vein thickening is caused by differentiation of intervein cells as veins (De Celis 2003; Sotillos and De Celis 2005; O'Connor *et al.* 2006). To determine whether loss of dMAN1 altered Dpp signaling, we examined the distribution of phospho-Mad in wild-type and *dMAN1* mutant wing imaginal discs (Figure 5A). We find that the overall level and pattern of phospho-Mad is changed in *dMAN1* mutants, wherein phospho-Mad localizes within a broader domain in the future wing blade and in the presumptive notum. These studies are consistent with the proposal that in the absence of dMAN1, Dpp signaling is increased. In addition to wing patterning differences, *dMAN1* mutants had held-out wings. Dissection of the indirect flight muscles showed that muscle structure is not grossly altered in *dMAN1* mutants (data not shown), suggesting that the held-out wings are not due to the disruption of the thoracic musculature (Baehrecke 1997; Lo and Frasch 1997; Sherwood *et al.* 2004). We noted that when *dMAN1* flies were left uncontained, they walked and jumped, but did not fly away. We used climbing assays to assess whether *dMAN1* mutants showed locomotion defects. Flies were placed in a graduated cylinder and tapped to the bottom, and the number of

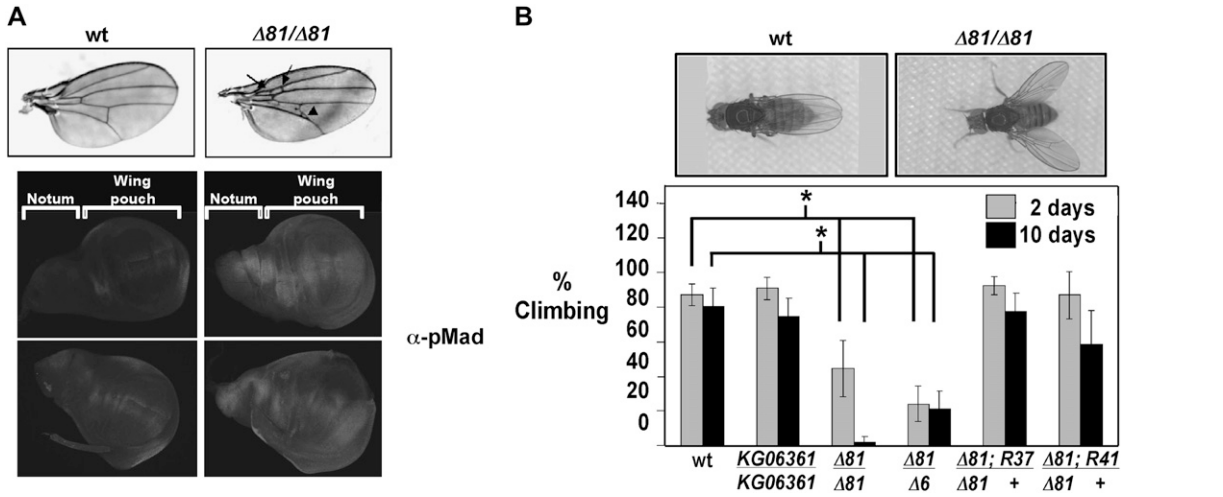

FIGURE 5.—Phenotypes associated with the loss of *dMAN1*. (A) Patterning defects associated with *dMAN1* mutants. Top, wing blades isolated from wild-type (wt, y^1w^{67}) and $dMAN1^{\Delta 81}$ homozygotes ($\Delta 81/\Delta 81$). Patterning defects include thickening of the longitudinal veins, an increased number of crossveins (arrows), and branching of the posterior crossvein (arrowhead). Bottom, wing imaginal discs isolated from wt and $\Delta 81/\Delta 81$ third instar larvae and stained with anti-phospho-Mad antibodies. Regions of the disc giving rise to the presumptive notum and wing blade are marked (B) Wing positioning and locomotion defects in *dMAN1* mutants. Top, comparison of wing positioning in wt and $\Delta 81/\Delta 81$ adults. Bottom, climbing assays detect locomotion defects in *dMAN1* mutants. The percentages of flies that climbed 30 cm in 1 min assayed at ages of 2 (shaded bars) and 10 (solid bars) days are shown. The genotypes tested were $\Delta 81/\Delta 81$, wt (y^1w^{67}), the parental *P* line (*KG06361*), the interallelic combination $\Delta 81/\Delta 6$, and $\Delta 81/\Delta 81$ carrying the *P[dMAN1-gen]* genomic rescue constructs, *R37* and *R41*. Error bars represent the mean plus or minus standard deviations. $^*P < 0.0001$ for $\Delta 81/\Delta 81$ relative to wild type and *KG06361*.

flies climbing to 30 cm in 1 min was recorded. These studies showed that 90% of the wild-type and parental *KG06361* flies reached 30 cm in 1 min, regardless of age. In contrast, 2-day-old $dMAN1^{\Delta 81}$ homozygotes and $dMAN1^{\Delta 81}/dMAN1^{\Delta 6}$ *trans*-heterozygotes climbed shorter distances, with only 25–40% of flies reaching the 30-cm mark in the same time period (Figure 5B). Interestingly, these climbing defects became more pronounced in 10-day-old adults, demonstrating age-enhanced phenotypes.

Tests of the fertility of *dMAN1* mutants showed that both sexes were affected. Males were sterile and females showed reduced fecundity (Figure 6, Table 3). Reproductive tissues from males and females were analyzed to understand these defects. $dMAN1^{\Delta 81}$ males had small, disorganized reproductive tissues that contained motile sperm (Figure 6), suggesting that sterility might reflect a failure of sperm delivery. Interestingly, in females, ovary defects were age dependent. The ovary is divided into ovarioles, each containing an assembly line of egg chambers with increasingly mature stages of development. Each egg chamber contains 16 interconnected germ-line cells, consisting of 15 nurse cells and one oocyte. DNA staining of ovaries isolated from 3-day-old $dMAN1^{\Delta 81}$ females showed ovarioles containing egg chambers representing all stages of oogenesis, with each carrying appropriate numbers of polyploid nurse and oocyte cells. We did note, however, that the $dMAN1^{\Delta 81}$ mutant ovaries had an increased number of stage 8 egg chambers with condensed DNA in the nurse cell nuclei, suggestive of apoptosis (Figure 6). TUNEL labeling confirmed DNA fragmentation in these ovaries (data not shown), indicating increased activation of the midstage programmed cell death response (BUSZCZAK and COOLEY 2000; MCCALL 2004). Ovaries dissected from 10-day-old $dMAN1^{\Delta 81}$ females lacked early stage egg chambers, instead showing an accumulation of stage 14 oocytes, indicative of a possible failure in egg deposition. These morphological changes were associated with age-dependent changes in female fecundity in comparison to wild-type controls (Table 3). Taken together, these data suggest that $dMAN1^{\Delta 81}$ females may have difficulty in egg deposition that results in a loss of early stages of oogenesis.

DISCUSSION

LEM-D proteins represent a family that shares an ~40-amino-acid BAF interacting domain. These proteins contribute to the function of the nuclear lamina, as most are enriched at the nuclear periphery, with several members embedded in the inner nuclear envelope. The human genome contains at least seven genes that encode >12 LEM-D proteins (MANILAL *et al.* 1996; DECHAT *et al.* 2000; LEE and WILSON 2004; BRACHNER *et al.* 2005). In comparison, the Drosophila genome contains five genes that encode 7 LEM-related proteins (Figure 1) (WAGNER *et al.* 2004a, 2006). Of these, 5 proteins contain domains that bind dBAF (supplemental Figure 1). These data suggest that the Drosophila

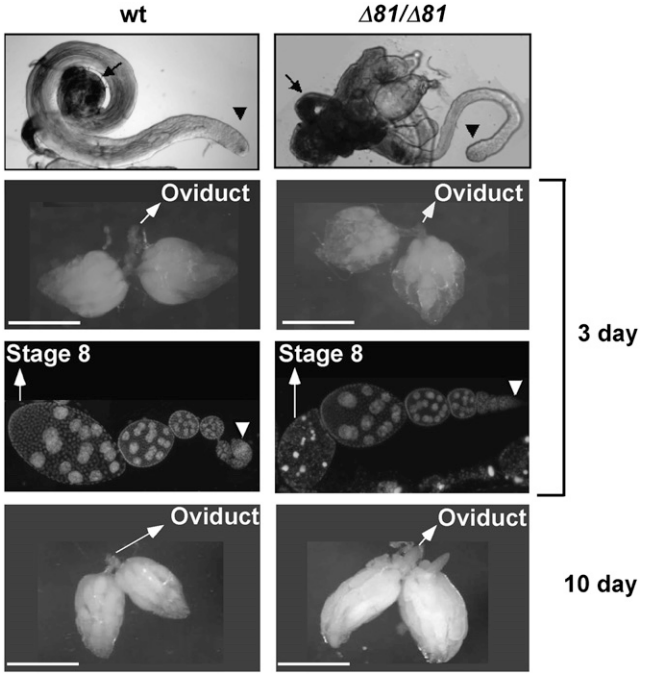

FIGURE 6.—Analysis of reproductive tissues of *dMAN1* mutants. Reproductive tissues of wild-type (wt, y^1w^{67}) and Δ*81*/Δ*81* male and female adults were isolated. Top, Δ*81*/Δ*81* males' reproductive tissues are structurally disorganized, including the seminal vesicle and accessory gland (arrow). The arrowhead indicates the tip of the testis that contains germ-line stem cells. Middle, ovaries dissected from 3-day-old wt and Δ*81*/Δ*81* females are morphologically similar. Staining DNA with DAPI detected an increase in the number of stage 8 egg chambers that showed intensely stained foci, suggestive of apoptosis. The arrowhead indicates the germarium. Bottom, ovaries dissected from 10-day-old Δ*81*/Δ*81* females with accumulation of stage 14 oocytes. The location of the oviduct is indicated. Bar, 1 mm.

LEM-D family is less complex than the vertebrate family and is comparable in size to the three-member family in *Caenorhabditis elegans* (STANKUNAS *et al.* 1998; LIU *et al.* 2003).

The Drosophila MAN1 protein: Vertebrate genomes carry two *MAN1*-related genes, one that encodes MAN1 and one that encodes LEM2. Several structural domains have been identified in hMAN1, including the amino-terminal LEM and lamina interacting domains, two transmembrane domains, and carboxyl-terminal MSC and UHM/RRM domains. LEM2 shows 83% identity with hMAN1, but lacks the carboxyl-terminal UHM/RRM (BRACHNER *et al.* 2005). Bioinformatic analysis of the Drosophila genome indicates that the annotated gene *CG3167* is the homolog of both proteins (WAGNER *et al.* 2006). Our analyses extend the recognized homology of the Drosophila and vertebrate MAN1 proteins. We find that amino acid sequences located between the transmembrane domains are conserved (supplemental Table 1). As this region resides in the perinuclear space, these findings indicate that dMAN1 might have some functions outside of the nucleus. Yeast two-hybrid studies demonstrated that the amino acid conservation in dMAN1 is functionally relevant, as several protein interaction partners are conserved with vertebrate homologs (Table 1).

TABLE 3

Analysis of fecundity of $dMAN1^{\Delta 81}$ females

	Age of females			
	3 days		10 days	
Genotype	No. females tested	No. eggs/female/day	No. females tested	No. eggs/female/day
yw;+/+	180	74.4	60	72.0
yw;Δ81/Δ81	1211	17.3	350	0.5

Mutational analyses of *dMAN1*: *dMAN1* is globally expressed, with the highest protein accumulation during embryogenesis, reflecting both maternal and zygotic contributions of protein (WAGNER *et al.* 2006). To understand the role of dMAN1 during Drosophila development, we generated mutant alleles by *P*-element mobilization. The excision-generated $dMAN1^{\Delta 81}$ allele carries a deletion of the *dMAN1* gene that starts upstream of the translation start codon and extends to the end of the coding region, indicating that $dMAN1^{\Delta 81}$ is a null allele. Homozygous $dMAN1^{\Delta 81}$ flies survive, but at a reduced level (~65%; Table 2). Similar findings were obtained in genetic studies of the single *C. elegans* homolog of MAN1, Ce-LEM2, where knockdown caused a slight reduction in viability (LIU *et al.* 2003). The survival of $dMAN1^{\Delta 81}$ homozygotes demonstrates that the Drosophila homolog of MAN1 and LEM2 is not essential for cell viability.

Analyses of a gene-trap allele of mouse *Man1*, $Man1^{GT}$, showed that homozygous mutants die during embryogenesis (ISHIMURA *et al.* 2006; COHEN *et al.* 2007). The phenotypes of $Man1^{GT}$ embryos suggest that lethality was due to hyperactivation of the TGF-β/BMP signaling pathway, resulting in an increased nuclear accumulation of phosphorylated Smad2/3 and changes in Smad target gene expression that increase extracellular matrix deposition and altered vascular remodeling. Our studies showing interactions between dMAN1 and the Drosophila R-Smads imply that dMAN1 might modulate the equivalent signaling pathway in flies (Table 1). This connection is supported by two additional observations. First, the wing phenotypes associated with homozygous $dMAN1^{\Delta 81}$ adults are reminiscent of altered Dpp signaling (RAFTERY and SUTHERLAND 1999; O'CONNOR *et al.* 2006). These mutant adults show held-out wings, as observed for some *dpp* mutants (GELBART 1982). Dissected wings show thickening of all longitudinal veins, variable numbers of additional anterior crossveins, and a broadening of the posterior crossvein (Figure 5). These phenotypes share features of those produced by

overexpression of Dpp in the pupal veins (Sotillos and De Celis 2005). Second, increased accumulation of phospho-Mad is observed in *dMAN1*$^{\Delta 81}$ mutant wing discs (Figure 5). Taken together, these data support evolutionary conservation of a role for dMAN1 in modulating the TGF-β/BMP signaling pathway.

As Dpp plays a central role in embryogenesis (Raftery and Sutherland 1999; Affolter *et al.* 2001; O'Connor *et al.* 2006), it seemed surprising that *dMAN1* null mutants survive to adulthood. We predicted that the large amounts of maternally supplied dMAN1 protein might compensate for the absence of zygotically produced product. Consistent with this proposal, mutant embryos produced from homozygous *dMAN1*$^{\Delta 81}$ females mated to *dMAN1*$^{\Delta 81}$/*CyO* males hatch at a lower frequency (~8%) relative to their heterozygous siblings (Figure 3). Surprisingly, a few of the hatched *dMAN1*$^{\Delta 81}$ larvae survived to adulthood, without any maternally or zygotically supplied dMAN1 (Figure 3). These observations suggest that misregulation of the TGF-β/BMP signaling pathway might be compensated for in Drosophila. This postulate is supported by previous observations that four copies of the *dpp* gene produced viable adults, even though dramatic changes in the dorsal–ventral patterning of the embryo occurred (Wharton *et al.* 1993).

LEM domain proteins show distinct developmental requirements: *dMAN1*$^{\Delta 81}$ adults displayed developmental defects affecting multiple tissues, such as the wing and male and female reproductive tissues (Figures 5 and 6). These mutants live as long as wild-type siblings and show no gross morphological defects in other tissues. Interestingly, we found that the locomotion and egg-laying defects were more severe in older flies, demonstrating an age-enhanced dysfunction of the nuclear lamina. Such findings are reminiscent of laminopathy patients, suggesting that studies of LEM domain proteins in Drosophila will provide insights into disease mechanisms. The tissue-specific phenotypes associated with loss of dMAN1 occur without detectable disturbances in nuclear structure.

Genetic analyses of a second Drosophila LEM domain gene, *otefin*, have been recently reported (Jiang *et al.* 2008). These studies showed that *otefin* is a nonessential gene, with flies showing developmental defects that are limited to the female germ line. Otefin is critical for germ-line stem cell (GSC) maintenance in females, through a role in transcriptional silencing of *bam*, a gene required for cystoblast differentiation. Repression is associated with a direct interaction between Otefin and Medea/Smad4, the Drosophila co-Smad, providing a second link between the Dpp/BMP signaling pathway and LEM domain proteins in Drosophila. Although dMAN1 is present in ovaries, loss of this protein affects later stages of oogenesis. These data, coupled with the different phenotypic consequences of loss of dMAN1 and Otefin, suggest that LEM-D proteins have distinct developmental requirements in the nuclear lamina.

One proposed role of LEM-D proteins is the regulation of nuclear envelope association of BAF. Genetic studies have shown that *dBAF* is essential for viability, with null mutants displaying a typical mitotic phenotype (Furukawa *et al.* 2003). That the loss of neither dMAN1 nor Otefin (Jiang *et al.* 2008) is lethal suggests one of two possibilities. Either Drosophila LEM-D proteins share roles in regulating BAF nuclear envelope interactions or dBAF has LEM-independent functions that are essential for survival. The former postulate is consistent with the demonstration that *C. elegans* LEM-D proteins share functions (Liu *et al.* 2003). While knockdown of Ce-emerin had no discernible effect on viability, knockdown of both Ce-LEM2 and Ce-emerin caused 100% lethality at the 100-cell stage. Further studies are needed to determine whether one or more LEM-D proteins have overlapping functions within the Drosophila nuclear lamina.

The nuclear lamina has been implicated in many processes, including control of nuclear shape and stability, nuclear anchoring and migration, DNA replication, regulation of gene expression, and chromatin organization (Wilson *et al.* 2001; Gruenbaum *et al.* 2005; Somech *et al.* 2005; Worman 2005; Bengtsson 2007). Homozygous *dMAN1*$^{\Delta 81}$ mutants are viable, suggesting that dMAN1 is not critical for DNA replication. Further, loss of dMAN1 has no obvious effects on nuclear shape or organization (Figure 4). These data indicate that the tissue-specific defects associated with *dMAN1* mutants may represent alterations in processes that include nuclear import or export or regulation of gene expression. Mechanisms describing how LEM-D proteins contribute to transcriptional regulation remain unclear. dMAN1 shows conservation in the LEM and MSC domains, supporting a role for dMAN1 in association with genes at the nuclear periphery, a zone commonly considered to be repressive to transcription (Wallrath *et al.* 2004; Gilbert *et al.* 2005). Additionally, vertebrate MAN1 antagonizes R-Smad signaling, a process that requires nuclear envelope placement (Osada *et al.* 2003; Raju *et al.* 2003; Hellemans *et al.* 2004; Pan *et al.* 2005). As dMAN1 associates with Smads and loss of this protein increases phospho-Smad accumulation (Figure 5, Table 1), these observations indicate that dMAN1 may have a conserved role in attenuation of nuclear signaling events, leading to changes in gene expression.

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