Unique and Shared Functions of Nuclear Lamina LEM Domain Proteins in *Drosophila*

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ABSTRACT The nuclear lamina is an extensive protein network that contributes to nuclear structure and function. LEM domain (*L*AP2, *e*merin, *M*AN1 domain, LEM-D) proteins are components of the nuclear lamina, identified by a shared ~45-amino-acid motif that binds Barrier-to-autointegration factor (BAF), a chromatin-interacting protein. *Drosophila melanogaster* has three nuclear lamina LEM-D proteins, named Otefin (Ote), Bocksbeutel (Bocks), and dMAN1. Although these LEM-D proteins are globally expressed, loss of either Ote or dMAN1 causes tissue-specific defects in adult flies that differ from each other. The reason for such distinct tissue-restricted defects is unknown. Here, we generated null alleles of *bocks*, finding that loss of Bocks causes no overt adult phenotypes. Next, we defined phenotypes associated with *lem-d* double mutants. Although the absence of individual LEM-D proteins does not affect viability, loss of any two proteins causes lethality. Mutant phenotypes displayed by *lem-d* double mutants differ from *baf* mutants, suggesting that BAF function is retained in animals with a single nuclear lamina LEM-D protein. Interestingly, *lem-d* double mutants displayed distinct developmental and cellular mutant phenotypes, suggesting that *Drosophila* LEM-D proteins have developmental functions that are differentially shared with other LEM-D family members. This conclusion is supported by studies showing that ectopically produced LEM-D proteins have distinct capacities to rescue the tissue-specific phenotypes found in single *lem-d* mutants. Our findings predict that cell-specific mutant phenotypes caused by loss of LEM-D proteins reflect both the constellation of LEM-D proteins within the nuclear lamina and the capacity of functional compensation of the remaining LEM-D proteins.

THE nuclear lamina is an extensive protein network underlying the nuclear envelope. This network is composed of the nucleus-specific intermediate filament proteins, the A- and B-type lamins, which form a structural platform for association of >200 proteins (Schirmer *et al.* 2003; Korfali *et al.* 2010; Malik *et al.* 2010). LEM domain (LAP2, emerin, MAN1 domain, LEM-D) proteins represent one family of lamin interacting proteins. This family shares an ~45-residue bihelical domain that was first identified in *L*AP2, *e*merin, and *M*AN1 (Lin *et al.* 2000; Mansharamani and Wilson 2005; Wagner and Krohne 2007). LEM-D proteins interact with the small, conserved protein called Barrier-to-autointegration factor (BAF), a protein that binds double-strand 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 to the nuclear lamina, thereby contributing to global nuclear organization.

Metazoan genomes encode several LEM-D proteins (Lee and Wilson 2004; Berk *et al.* 2013). Most show enriched localization within the nuclear lamina, wherein the LEM-D proteins direct shared protein associations. For example, emerin and MAN1 interact with each other, as well as with the A- and B-type lamins and transcriptional regulators, such as the Germ-cell-less repressor (Mansharamani and Wilson 2005). In addition, LEM-D proteins direct unique interactions. For example, MAN1 associates with the receptor regulated Smads (Osada *et al.* 2003; Raju *et al.* 2003; Pinto *et al.* 2008; Wagner *et al.* 2010), the downstream effectors of the TGF-β signaling pathways, while emerin associates with β-catenin (Markiewicz *et al.* 2006), a mediator of Wnt signaling. Recent evidence suggests that some LEM-D proteins are not enriched in the nuclear lamina, but shuttle

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between the cytoplasm and nucleus. These proteins, exemplified by LEM-3/Ankle1, are distinguished by the presence of a C-terminal GIY–YIG motif associated with endonuclease activity (Brachner *et al.* 2012) and hypersensitivity to DNA damage (Dittrich *et al.* 2012). Together, these findings emphasize that LEM-D proteins have the capacity to make diverse contributions to many nuclear processes, ranging from transcriptional regulation to maintenance of genome stability.

Mutations in genes encoding the nuclear lamina LEM-D proteins cause several human diseases. The loss of LEM-D proteins is associated with bone density disorders, cardiomyopathies, and muscular dystrophies (Worman *et al.* 2010). These diseases display age-enhanced, tissue-restricted pathology, even though LEM-D proteins are globally expressed. The basis for tissue-restricted defects is unknown. One possibility is that the affected tissues require a function unique to one member of the LEM-D family. Alternatively, cell types may have differences in the abundance of LEM-D proteins (Solovei *et al.* 2013), which may predispose cells to loss of a disease-associated protein. Distinguishing between these possibilities requires an improved understanding of the functions of LEM-D proteins and their capacity for regulatory compensation.

Drosophila melanogaster serves as an excellent model to study how nuclear lamina components contribute to tissue-specific regulation during development. This model has been powerful in defining effects of novel disease-causing mutations in the human A-type lamin (Dialynas *et al.* 2012), demonstrating that some disease-causing mutations cause a loss of nuclear compartmentalization of lamins and associated proteins, which may contribute to muscle disease. *Drosophila* encodes a three-member nuclear lamina LEM-D family (Figure 1A). These LEM-D proteins include dMAN1, Otefin (Ote), and Bocksbeutel (Bocks) (Ashery-Padan *et al.* 1997a,b; Wagner *et al.* 2004, 2006; Pinto *et al.* 2008). dMAN1 represents the *Drosophila* homolog of LEM2 and MAN1, whereas Ote and Bocks are predicted to be homologs of emerin (Wagner *et al.* 2006; Wagner and Krohne 2007). Genetic analyses have demonstrated that both dMAN1 and Ote are required for *Drosophila* development. Loss of dMAN1 causes a moderate decrease in adult viability, with surviving adults showing male sterility, decreased female fertility due to egg retention, defects in neuromuscular junctions, flightlessness associated with altered wing patterning and positioning, and locomotion difficulties (Pinto *et al.* 2008; Wagner *et al.* 2010). In contrast, phenotypes associated with $ote^{-/-}$ mutants are restricted to the ovary, where germline stem cells (GSCs) show age-enhanced loss (Jiang *et al.* 2008; Barton *et al.* 2013). The nonoverlapping defects associated with Ote and dMAN1 loss imply that these proteins make unique regulatory contributions during development.

The *Drosophila* nuclear lamina LEM-D proteins share extensive homology within the LEM-D (Figure 1B). However, outside of this domain, homology is minimal. Non-LEM-D regions in Ote and Bocks are predicted to be largely disordered, a feature shared with emerin (Wolff *et al.* 2001), whereas dMAN1 contains several defined domains (Wagner *et al.* 2006; Pinto *et al.* 2008). Even though homology is limited, non-LEM-D regions of the *Drosophila* LEM-D proteins direct common protein associations, such as interactions with the A- and B-type lamins, Lamin C and lamin Dm_0, respectively (Pinto *et al.* 2008; Schulze *et al.* 2009). These shared protein partners imply that *Drosophila* LEM-D proteins possess overlapping functions, as found for LEM-D proteins in other organisms (Gruenbaum *et al.* 2002; Liu *et al.* 2003; Huber *et al.* 2009; Barkan *et al.* 2012; Gonzalez *et al.* 2012; Reil and Dabauvalle 2013). However, the extent of any regulatory compensation among the three *Drosophila* nuclear lamina LEM-D proteins is unknown. To begin to address this question, we generated mutations in the *bocks* gene. Analyses of animals carrying *bocks* null alleles demonstrated that complete loss of Bocks causes no overt developmental defects. Next, we generated animals carrying null alleles of two *lem-d* genes. We found that all *lem-d* double mutants fail to survive to adulthood, demonstrating that *Drosophila* LEM-D proteins are functionally redundant. Analyses of the developmental and cellular phenotypes in *lem-d* double mutants revealed distinct mutant phenotypes, implying that pairs of LEM-D proteins share different functions. Further, *lem-d* double mutants display phenotypes distinct from *baf* mutants, indicating that essential functions of LEM-D proteins extend beyond BAF regulation. Finally, we determined whether ectopic production of individual LEM-D proteins rescued tissue-restricted phenotypes found in the $ote^{-/-}$ and $dMAN1^{-/-}$ mutants. We found that production of Bocks, but not dMAN1, rescued $ote^{-/-}$ sterility, demonstrating that Ote and Bocks are functional homologs. In contrast, neither Bocks nor Ote rescued $dMAN1^{-/-}$ mutant phenotypes, implying that dMAN1 makes unique contributions to wing development. Based on our studies, we predict that cell-specific mutant phenotypes resulting from loss of individual LEM-D proteins depend upon two factors. These include the presence of other LEM-D proteins within the nuclear lamina and the capacity of remaining LEM-D proteins to functionally compensate for the lost protein.

Materials and Methods

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. Crosses were carried out in vials at 25°, unless otherwise noted. Stocks used in this study include y^1w^{67c23} (the wild-type reference control), two *dMAN1 alleles* [$dMAN1^{\Delta 81}$ and $dMAN1^{\Delta 26}$ (Pinto *et al.* 2008)], and two *ote* alleles [$ote^{B279\text{-}G}$ and ote^{halPk} (Barton *et al.* 2013)].

Generation of bocks deletion alleles

The parental line used to generate *bocks* mutants was $bocks^{CB03586}$, a line that carries a *white* marked *P* element

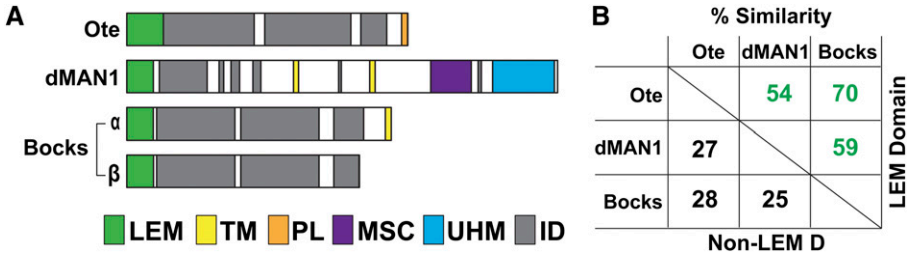

Figure 1 *Drosophila* nuclear lamina LEM-D proteins. (A) Three genes encode nuclear lamina-enriched LEM-D proteins, *otefin* (*ote*), *dMAN1*, and *bocksbeutel* (*bocks*). All of the encoded proteins carry an amino-terminal LEM-D (green box). The *bocks* gene encodes two isoforms. The Bocks α- and β-isoforms are distinguished by a unique 50-amino-acid carboxyl-terminal domain in Bocks α-isoform that carries a transmembrane domain (yellow boxes). Outside of the LEM-D, the three proteins carry regions predicted to be intrinsically disordered (ID, gray boxes) and additional domains, including the MAN1–Src1-p C-terminal domain (MSC, purple box), the U2AF homology motif (UHM, blue box), a transmembrane domain (TM, yellow box) or a peripheral localization domain (PL, orange box). (B) Shown is a chart of the percentage similarity derived from pairwise alignments of amino acid sequences of LEM-D proteins, including either the LEM-D alone or the non-LEM-D regions of the proteins. In these comparisons, the larger Bocks α-isoform was used.

[*P{PTT-GB}*] inserted into the 5′-untranslated region of the *bocks* gene (Buszczak *et al.* 2007). *P* mobilization was achieved using a chromosomal source of transposase, *P [ry$^+$ Δ2-3]* (99B) (Robertson *et al.* 1988). Homozygous *y^{1}w^{67c23}*; *bocks^{CB03586}* females were crossed to males *y$^+$w$^+$/Y*; *CyO/Sp*; *Δ2-3 Sb/TM6*, *Ubx* males. Single *y^1 w^{67c23}; bocks^{CB03586}/ Δ2-3 Sb* red-eyed males (indicating the presence of the *white* marked P element) were mated to females that were *y$^+$ w^{67c23}*; *bocks$^+$/TM6, Tb*. Excisions were identified as white-eyed, Tubby (*TM6*), non-Sb flies (*y$^+$ w^{67c23}/Y; bocks*/TM6, Tb*). DNA was isolated from these stocks and Southern analysis was completed to determine whether *P* excision resulted in deletions of DNA sequences containing the *bocks* gene. Two *bocks* deletion alleles were identified, *bocks^{Δ10}* and *bocks^{Δ66}* (Figure 2A). The endpoints of each deletion was determined using PCR to isolate an appropriately sized fragment from genomic DNA. These fragments were directly sequenced.

qPCR analysis of gene expression

Ten wandering, late third-instar larvae were collected, and frozen at −80° and RNA was isolated using TRIzol (Invitrogen). Total RNA was DNAse I treated using DNA-free (Ambion) and reverse transcribed using high-capacity cDNA kit with random hexamer primers (Applied Biosystems). Cycle threshold values were normalized to the housekeeping gene, *RpL32*. Fold-enrichment was calculated using the ΔΔCT method (Livak and Schmittgen 2001). Primer pairs for RNA quantification are listed 5′ to 3′ for *RpL32* (forward AAGATGACCATCCGCCCAGCATAC and reverse ACGCACTCTGTTGTCGATACCCTTG), *heat shock factor* (forward TTCTCAGCGCCACTGTATTT and reverse GATGTGCCCACCAACAGTA), *CG8312* Up (forward CAACACCTACAATGCCAAGAAG and reverse TCAGGGTTATGACGTTCGTG), and *CG8312* Dn (forward AGCCTCCTGCCCACTATTA and reverse CCGCATCTTTCTCATCGAACT).

Western and immunohistochemical analyses

Polyclonal goat anti-Otefin antibodies were generated against the amino-terminal 187 amino acids of Otefin. The coding sequence for these amino acids was amplified from *y^{1}w^{67c23}* genomic DNA and cloned into the pET21-a (Novagen) bacterial expression vector. The purified His-tagged Ote fragment was used for immunization in goats (Elmira Biologicals). The resulting serum was affinity purified using bacterially expressed, full length, His-tagged Otefin (Actigel, Sterogen).

Western analyses were completed on adult protein extracts. These proteins were separated on 10% SDS polyacrylamide gels and transferred to 0.2-μm nitrocellulose membrane at 70 V for 1 hr. After blocking in PBS with 5% milk, 0.1% Tween, membranes were incubated overnight in one of the following primary antibodies: guinea pig anti-Bocks [1:2,000; (Wagner *et al.* 2004)], mouse anti-lamin Dm_0 (DSHB ADL84.12, 1:1,000), mouse anti-Lamin C (DSHB LC28.26, 1:1,000), sheep anti-dMAN1 [1:50; (Pinto *et al.* 2008)], and goat anti-Otefin (1:1000). Primary antibodies were detected with HRP-conjugated secondary antibodies (1:20,000, Sigma) and detected by chemiluminescence (SuperSignal West Pico, Pierce). Blots were stripped by 30 min of agitation at 40° in 10% SDS, 1% β-mercaptoethanol in PBS and reblotted for anti-α-tubulin (Sigma, T5168, 1:20,000) to serve as a loading control.

Immunohistochemical staining of adult ovaries was performed as described in Baxley *et al.* (2011). Nonmuscle larval tissues were fixed in 4% EM-grade paraformaldehyde, followed by a 1-hr wash in 1% TritonX-100 in PBS. Larval body wall muscles were prepared for staining as described in Budnik *et al.* (1990). The following primary antibodies were used for immunohistochemical analyses: mouse anti-Lamin C (DSHB LC28.26, 1:200), rabbit anti-heterochromatic protein 1 (HP1, Covance PRB291C, 1:400), rabbit anti-lamin Dm_0 (Fisher, 1:200), mouse MAb414, which recognizes FG-repeat-containing nucleoporins (FG-Nups, Covance, 1:100 for nonmuscle tissue and 1:3000 for muscles), mouse anti-phosphohistone 3 (pHH3, Millipore 06-570, 1:200), rabbit anti-vasa (Santa Cruz sc-30210, 1:300), and mouse anti-spectrin (DSHB 3A9, 1:50).

Lethal phase analysis of lem-d double mutants

Heterozygous balanced *lem-d* double mutant stocks were generated. The genotypes of these stocks were (1) *y^{1}w^{67c23}; ote^{PK}/CyO-y$^+$; bocks^{Δ66}/TM6B-Tb, Hu*; (2) *y^{1}w^{67c23}; ote^{B279G}/CyO-y$^+$; bocks^{Δ10}/TM6B-Tb, Hu*; (3) *y^{1}w^{67c23}; ote^{PK}, dMAN1^{Δ26}/CyO-y$^+$*; (4) *y^{1}w^{67c23}; ote^{B279G}, dMAN1^{Δ81}/CyO-y$^+$*; (5) *y^{1}w^{67c23};*

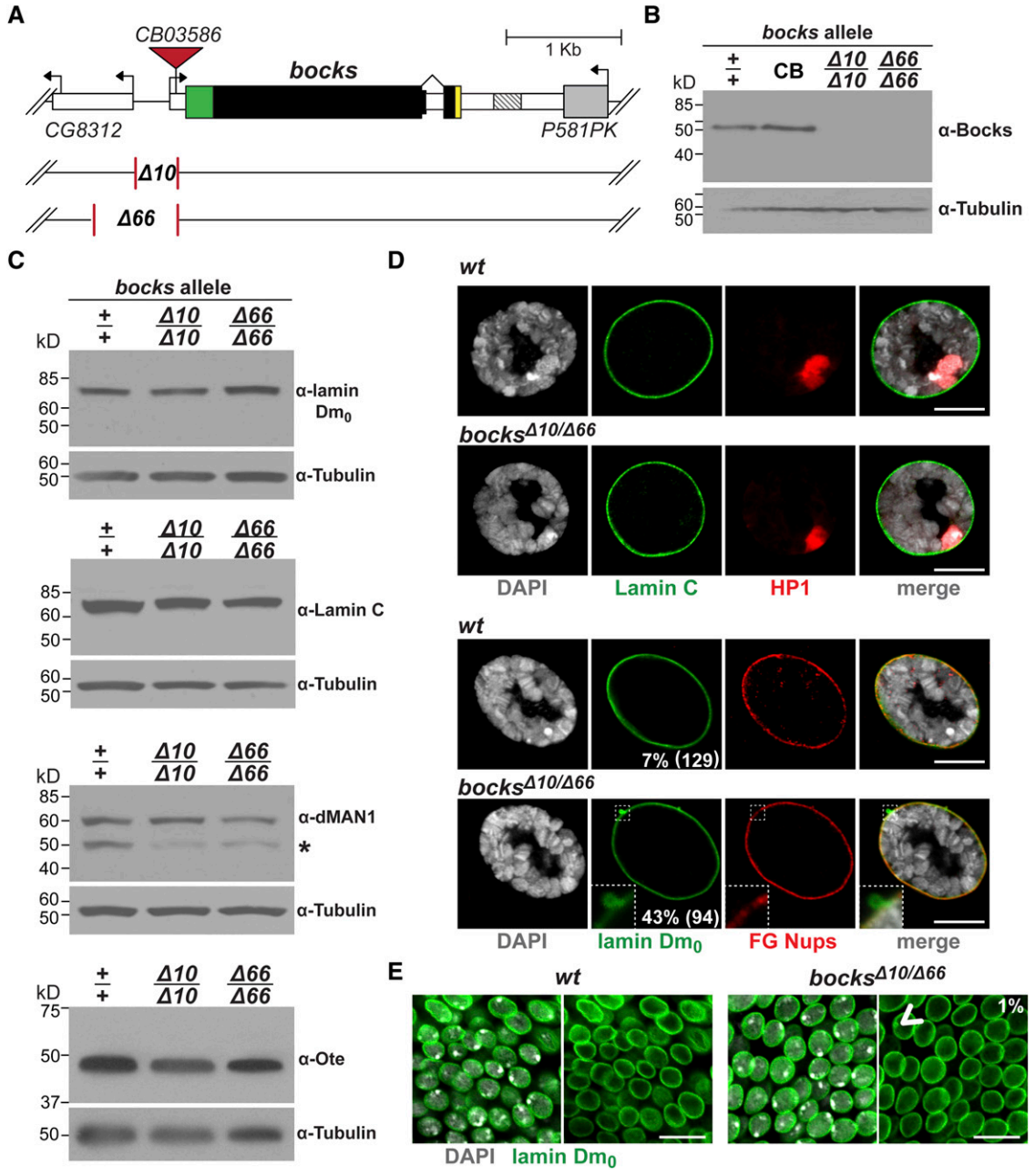

Figure 2 Generation and analysis of *bocks* deletion alleles. (A) Structure of the *bocks* locus, including a portion of the uncharacterized *CG8312* gene and the overlapping *P581PK* gene. The *bocks* coding region is shown as a large rectangle (black), depicting the location of the LEM-D (green) and the transmembrane domain (yellow). Deletion alleles of *bocks* were generated by imprecise deletion of a *P* element inserted at +11 in the $bocks^{CB03586}$ allele (inverted red triangle). The $bocks^{\Delta 10}$ has a 344-bp and $bocks^{\Delta 66}$ has a 728-bp deletion. (B)Western blot of protein extracts isolated from $bocks^{+/+}$ (+/+), $bocks^{CB03586}$ (CB) adults, and adults homozygous for one of the two *bocks* deletion alleles. The Western blot was probed with antibodies against Bocks and reprobed with antibodies against α-tubulin, which serves as a loading control. Note that our separation conditions detect only a single band, which likely represents both Bocks isoforms. (C) Shown are Western blots of protein extracts from $bocks^{+/+}$, $bocks^{\Delta 10/\Delta 10}$, $bocks^{\Delta 66/\Delta 66}$ adults. These blots were probed with antibodies against lamin Dm_0, Lamin C, dMAN1, or Ote and reprobed with antibodies against α-tubulin. (*) A breakdown product of dMAN1, which is primarily detected in $bocks^{+/+}$ extracts. (D) Top: confocal images of wild-type (wt) and *bocks* mutant third-instar larval salivary gland nuclei stained for DNA (DAPI), Lamin C (green), HP1 (red), and DAPI (gray), with the merged image shown on the right. Bottom: confocal images of wild-type (wt) and *bocks* mutant third-instar larval salivary gland nuclei stained for DNA (DAPI), lamin Dm_0 (green), FG-repeat containing nuclear porins (FG-Nups, red), and the merged image. The lamin Dm_0 staining shows O-shaped deformities in the $bocks^{-/-}$ nuclear lamina, evident in the higher magnification inset in the bottom left corner. The prevalence of any deformities, including solid lamin Dm_0 accumulations in $bocks^{+/+}$, is noted in the bottom right corner of lamin Dm_0 images. The total number of nuclei analyzed from a minimum of five animals is in parentheses. (E) Confocal images of ventral nerve cord cells from wild-type (wt) and $bocks^{-/-}$ mutant third-instar larvae stained for lamin Dm_0 (green) and DAPI (gray). These diploid cells show rare deformities (arrowhead) in the nuclear lamina, with a prevalence of ~1%. Genotypes are noted at the top. Scale bars, 10 μm.

dMAN1$^{\Delta 81}$*/CyO-y*$^{+}$*; bocks*$^{\Delta 10}$*/TM6B-Tb, Hu*; (6) *y*^{1}*w*^{67c23}*; dMAN1*$^{\Delta 26}$*/CyO-y*$^{+}$*; bocks*$^{\Delta 66}$*/TM6B-Tb, Hu*. Crosses between males and females from different balanced heterozygous stocks were set up in bottles. After an acclimation period, eggs were collected on yeasted orange juice plates for 24 hr and incubated at 25° for 24 hr. Hatched second-instar larval progeny were collected from these plates and genotyped, using the *yellow* marker to identify the second chromosome *CyO, y*$^{+}$ balancer, and the *Tubby* marker (Tb) to identify the third chromosome *TM6b, Tb, Hu* balancer. Heterozygous and homozygous *lem-d* mutants of distinct genotypes were placed into different vials and allowed to develop at 25°. After 2 weeks, the number of pupae and adults produced in each vial were scored, leading to determination of the percentage survival of *lem-d* mutants during larval and pupal stages of development.

Complementation by ectopic expression

For each nuclear lamina LEM-D protein, a heat-inducible expression transgene was constructed. The *lem-d* cDNAs were cloned downstream of the *heat shock 70* promoter present within a *white*-marked *P* transposon. In total, four expression transposons were made, including (1) *P[hsp70: ote]*, (2) *P[hsp70:dMAN1]*, (3) *P[hsp70:bocks α]*, and (4) *P[hsp70: bocks β]*. Lines carrying each *P[hsp70:lem-d]* transposon were generated by *P*-element-mediated germline transformation. To assess the capacity of ectopically produced LEM-D proteins to rescue mutant phenotypes associated with dMAN1 and Ote loss, we established stocks in which each *P[hsp70:lem-d]* transgene was crossed into *ote*$^{-/-}$ or *dMAN1*$^{-/-}$ mutant background. The resulting *y*^{1}*w*^{67c23}; *ote*$^{-}$*/CyO, y*$^{+}$; *P[hsp70:lem-d]* or *y*^{1}*w*^{67c23}; *dMAN1*$^{-}$*/ CyO, y*$^{+}$; *P[hsp70:lem-d]* flies were crossed to *y*^{1}*w*^{67c23}; *ote*$^{-}$*/CyO* or *y*^{1}*w*^{67c23}; *dMAN1*$^{-}$*/ CyO* flies to generate *lem-d*$^{-/-}$, *P[hsp70:lem-d]* progeny. After 2 days at 25°, progeny received heat shock daily in a 37° water bath for 1 hr and returned to incubation at 25° until eclosion. The emerged *lem-d*$^{-/-}$, *P[hsp70:lem-d]* adults were assessed for complementation of *dMAN1* mutant phenotypes by examination of wing patterning and complementation of *ote* mutant phenotypes by quantification of egg production and immunohistochemical analysis of ovaries.

Results

Generation of bocks mutants

To understand the functional relationship between the *Drosophila* nuclear lamina LEM-D proteins, we needed to generate mutations in the *bocks* gene, as none existed. This gene encodes two isoforms that differ in the amino acid composition of their carboxyl-terminal domains. The Bocks α-isoform is the larger isoform, carrying a transmembrane domain in its unique C terminus that targets this protein to the nuclear envelope. In contrast, the smaller Bocks β-isoform lacks a transmembrane domain and localizes primarily to the nucleoplasm (Wagner *et al.* 2004). The *bocks* gene resides in a gene dense region, located downstream of the divergently transcribed *CG8312* gene and overlapping with the *P58IPK* gene (Figure 2A). We generated mutations in the *bocks* gene through mobilization of a *P* transposon inserted within the 5′-untranslated region of the parental *bocks*^{CB03586} gene. This strategy produced two *bocks* deletion alleles, *bocks*$^{\Delta 10}$ and *bocks*$^{\Delta 66}$. Deletions in both alleles extend 5′ from +11, with *bocks*$^{\Delta 10}$ carrying a 344-bp deletion and *bocks*$^{\Delta 66}$ carrying a 728-bp deletion (Figure 2A). Western analysis revealed that both alleles fail to accumulate Bocks protein (Figure 2B), demonstrating that two null *bocks* alleles were generated.

The upstream *CG8312* gene resides close to *bocks*. Three promoters regulate *CG8312* transcription, positioned ~0.3, ~0.9, and ~11.8 kb upstream of the *bocks* transcription start site. Both *bocks* deletion alleles remove the first *CG8312* promoter. In *bocks*$^{\Delta 10/\Delta 10}$, 609 bp of 5′ sequences remain upstream of the second *CG8312* promoter, while in *bocks*$^{\Delta 66/\Delta 66}$, 225 bp remain. To address whether these deletions alter *CG8312* transcription, we isolated RNA from *bocks*$^{+/+}$, *bocks*$^{\Delta 10/\Delta 10}$, and *bocks*$^{\Delta 66/\Delta 66}$ late third-instar larvae and measured levels of *CG8312* RNA using quantitative real-time PCR (Supporting Information, Figure S1). Two primers sets quantified *CG8312* RNA, evaluating levels of RNA produced from all promoters or only the first two promoters. In *bocks*$^{\Delta 10/\Delta 10}$ larvae, *CG8312* RNA levels were unchanged, implying that the first promoter is not used during this stage of development. In *bocks*$^{\Delta 66/\Delta 66}$ larvae, *CG8312* RNA levels were reduced by 30%, due to decreased transcription from the second *CG8312* promoter. Taken together, these data suggest that phenotypes observed in *bocks*$^{\Delta 10/\Delta 10}$ or *bocks*$^{\Delta 10/\Delta 66}$ mutants are due to loss of Bocks function.

Once *bocks* mutations were available, we defined effects of Bocks loss on development. To this end, we crossed *y*^{1}*w*^{67c23}; *bocks*$^{\Delta 10}$*/Sb* and *y*^{1}*w*^{67c23}; *bocks*$^{\Delta 66}$*/Sb* females and males and determined the number of *bocks*$^{+/-}$ (Sb) and *bocks*$^{-/-}$ (non-Sb) progeny. We found that *bocks*$^{-/-}$ adults were obtained at the expected number, with these adults displaying normal morphology and fertility (data not shown). These experiments demonstrate that Bocks is not essential for *Drosophila* development. Previous studies showed that depletion of Bocks in cultured *Drosophila* cells had no effect on the formation of the nuclear lamina (Wagner *et al.* 2004). Similarly, we found that *in vivo* loss of Bocks did not alter the accumulation or localization of many nuclear lamina components (Figure 2, C and D). We found that localization of Lamin C, the FG-repeat nuclear pore proteins (FG-Nups), and Heterochromatin Protein 1 (HP1) was normal in *bocks*$^{-/-}$ third-instar larval tissues (Figure 2D), implying that Bocks is not required for general maintenance of the nuclear lamina structure. We did detect an abnormal lamin Dm_0 distribution in many *bocks*$^{-/-}$ salivary gland polytene nuclei (43% relative to 7% in *bocks*$^{+/+}$ salivary gland nuclei), but at a much lower level in diploid nuclei (1%). In the abnormal *bocks*$^{-/-}$ salivary gland nuclei, the lamin Dm_0 was found in one to seven O-shaped lamin Dm_0-containing structures, which lacked lamin C and DNA (Figure 2D). In contrast, the abnormal *bocks*$^{+/+}$ salivary

gland nuclei contained solid structures. Similarly, O-shaped structures were observed in salivary gland nuclei that express mutant forms of Lamin C, although the number of lamin aggregates in *bocks*$^{-/-}$ nuclei is lower (Schulze *et al.* 2005, 2009). These observations suggest that loss of Bocks may weaken the nuclear lamina in large nuclei, causing the irregularities found in salivary gland nuclei.

At least two LEM-D proteins are required for development

To understand redundancy among *Drosophila* nuclear lamina LEM-D proteins, we studied developmental and cellular changes associated with the simultaneous loss of pairs of LEM-D proteins. In all cases, homozygous *lem-d* double mutants were produced from crosses of balanced heterozygous *lem-d* double mutant males and females, generating offspring that carried heteroallelic combinations of mutant alleles of each *lem-d* gene. In this way, we eliminated confounding recessive effects of second-site mutations on homozygous mutant chromosomes.

To define the developmental potential of *lem-d* double mutants, we collected and genotyped second-instar larvae resulting from crosses of balanced heterozygous *lem-d* double mutants. These larvae were placed into separate vials and the percentage of larvae developing into pupae and adults was determined. As a control, we conducted the same experiment with second-instar larvae collected from crosses of balanced heterozygous single *lem-d* mutants. In each case, individual *lem-d* mutant second-instar larvae survived at levels similar to those of the wild-type control (Figure 3). In contrast, *lem-d* double mutants showed decreased survival. Several observations were made from these experiments. First, heterozygous combinations of some *lem-d* mutant alleles reduced adult survival (Figure S2A). For example, *ote*$^{+/-}$; *bocks*$^{+/-}$ and *dMAN1*$^{+/-}$; *bocks*$^{+/-}$ heterozygotes had reduced viability, while *ote*$^{+/-}$, *dMAN1*$^{+/-}$ heterozygotes had wild-type survival (Figure S2A). Further, *dMAN1*$^{-/-}$; *bocks*$^{+/-}$ adults were never recovered, even though *dMAN1*$^{-/-}$, *ote*$^{+/-}$ adults were recovered at nearly the same level as *dMAN1*$^{-/-}$ single mutants (Figure S2B). These observations suggest that Bocks has a critical role in buffering the developmental consequences resulting from loss of dMAN1 or Ote. Second, no homozygous *lem-d* double-mutant adults were recovered (Figure 3). These data demonstrate that at least two nuclear lamina LEM-D proteins are needed for development. Interestingly, pairs of homozygous *lem-d* double mutants displayed distinct stages of lethality. While only ~10% of the *ote*$^{-/-}$; *bocks*$^{-/-}$ second-instar larvae formed pupae, ~60% of *dMAN1*$^{-/-}$, *ote*$^{-/-}$ and ~55% of *dMAN1*$^{-/-}$; *bocks*$^{-/-}$ second-instar larvae formed pupae (Figure 3). Together, these experiments reveal that pairs LEM-D proteins share distinct developmental functions, which are essential for viability. We suggest that the shared function(s) of Ote and Bocks are needed during larval stages of development, whereas the critical function(s) that Ote and Bocks share with dMAN1 are needed during pupal development.

All LEM-D proteins interact with BAF (Segura-Totten and Wilson 2004; Pinto *et al.* 2008). We were interested in understanding whether the lethality associated with *lem-d* double mutants was linked to altered BAF function. Previous studies showed that *baf*$^{-/-}$ mutants fail to develop beyond the larval–pupal transition (Furukawa *et al.* 2003). In *baf*$^{-/-}$ larvae, endocyclic larval tissues that undergo DNA replication without cell division were of a normal size, but mitotically active tissues, such as brains and imaginal discs were either small or absent, respectively. Further, nuclei in *baf*$^{-/-}$ tissues were misshapen, showing a convoluted nuclear lamina and intranuclear lamin Dm_0 accumulation (Furukawa *et al.* 2003). We reasoned that if *lem-d* double-mutant lethality were related to loss of BAF function, then *lem-d* double mutants would show *baf* mutant phenotypes. First, we compared development of *lem-d* double mutants with *baf* mutants. Our studies revealed that *ote*$^{-/-}$; *bocks*$^{-/-}$ mutants die earlier than *baf*$^{-/-}$ mutants, while survival of *dMAN1*$^{-/-}$, *ote*$^{-/-}$ and *dMAN1*$^{-/-}$; *bocks*$^{-/-}$ mutants were similar (Furukawa *et al.* 2003). The earlier lethality of *ote*$^{-/-}$; *bocks*$^{-/-}$ mutants implies that the critical function(s) shared by Ote and Bocks extend beyond BAF regulation. Second, we assessed cell proliferation of *lem-d* double-mutant brain and in imaginal discs that ultimately form adult tissues. Proliferation was assessed using an antibody against phosphorylated serine 10 of histone H3 (pHH3), a selective marker for mitosis (Hendzel *et al.* 1997). Unlike *baf*$^{-/-}$ mutants, the *ote*$^{-/-}$, *dMAN1*$^{-/-}$ and the *dMAN1*$^{-/-}$; *bocks*$^{-/-}$ larvae had normal sized brains and imaginal discs with extensive pHH3 staining (Figure 4). However, similar to *baf*$^{-/-}$ (Furukawa *et al.* 2003), *ote*$^{-/-}$; *bocks*$^{-/-}$ larval brains were small with little pHH3 staining, and imaginal disc tissues were small or absent (Figure 4). These experiments indicate that cells require either Ote or Bocks for mitotic growth. Third, we stained *lem-d* double-mutant tissues with antibodies against lamins, HP1, and the FG-Nups to examine nuclear organization. In most *lem-d* double-mutant tissues, nuclei had a uniform nuclear lamina and normal HP1 localization (Figure 5). Again, salivary gland nuclei were exceptional, wherein *bocks*$^{-/-}$ double mutants had nuclei with an abnormal localization of lamin Dm_0 (Figure 5A). Additionally, many *ote*$^{-/-}$, *dMAN1*$^{-/-}$ nuclei (~20%) had abnormal lamin Dm_0 structures (Figure 5A), although these were solid structures instead of the O-shaped structures found in *bocks*$^{-/-}$ mutants. These observations reinforce the proposal that giant polyploid nuclei are sensitive to loss of nuclear lamina components. Even so, no cytoplasmic or intranuclear accumulation of lamins or FG-Nups was observed, implying that the nuclear lamina is largely maintained in *lem-d* double mutants. Taken together, our data suggest that mutant phenotypes associated with loss of two LEM-D proteins do not match those found with loss of BAF. Based on the lack of aligned phenotypes, we conclude that loss of BAF function may not be a primary cause of lethality in *lem-d* double mutants.

LEM-D proteins directly interact with Lamin C and lamin Dm_0 (Pinto *et al.* 2008; Schulze *et al.* 2009). Previous

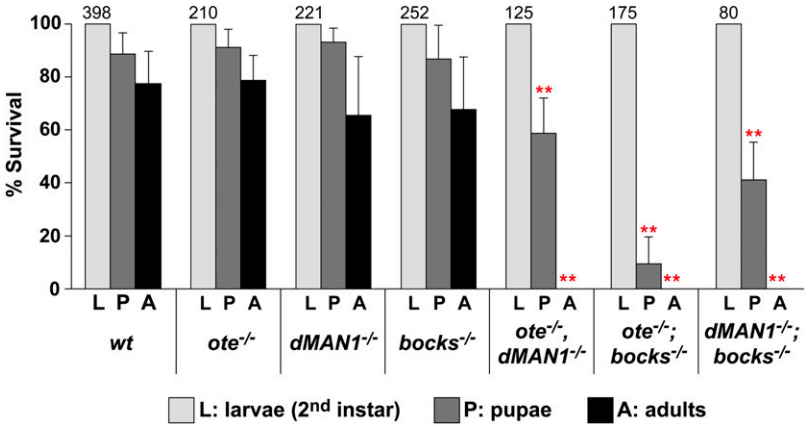

Figure 3 At least two nuclear *Drosophila* LEM-D proteins are required for adult survival. Shown is a graph of the percent survival of collected second-instar larvae (L) to pupae (P) and adults (A) of the indicated genotypes, including the following combinations of *lem-d* alleles: $ote^{B279G/PK}$, $dMAN1^{\Delta 81/\Delta 26}$, and $bocks^{\Delta 10/\Delta 66}$. The total number of second-instar larvae analyzed is listed above each set of bars. At least three independent experiments were completed, with error bars corresponding to standard deviation. Student's *t*-test compared the percentage survival of *lem-d* double mutants and the wild-type control at the matching developmental stages (* $P < 0.05$, ** $P < 0.01$).

studies revealed that muscle-specific production of mutant Lamin C isoforms causes semilethality, with body wall muscles in larvae showing the formation of cytoplasmic aggregates of nuclear lamina components, such as the FG-Nups (Dialynas *et al.* 2012). We wondered whether lethality of *lem-d* double mutants might be similarly related to muscle-specific dysfunction. For this reason, we stained body wall muscles in *lem-d* double mutants with antibodies against the FG-Nups. We found that mutant nuclei had uniform nuclear lamina, with no evidence of cytoplasmic aggregates (Figure 5C). These studies suggest that LEM-D proteins are not required for maintenance of the nuclear lamina in muscle nuclei. Larval body wall muscles form during embryogenesis when maternally contributed LEM-D proteins are present. Thus, it remains possible that LEM-D proteins contribute to the formation of nuclear lamina in muscle nuclei.

Increased expression of Bocks rescues GSC loss in ote$^{-/-}$ ovaries

Loss of dMAN1 or Ote causes tissue-restricted adult phenotypes (Jiang *et al.* 2008; Pinto *et al.* 2008; Wagner *et al.* 2010; Barton *et al.* 2013). We reasoned that such phenotypes might result because of cell-specific differences in the nuclear lamina, wherein affected cells may have insufficient amounts of a compensating LEM-D protein. To test this prediction, we determined whether the *dMAN1*$^{-/-}$ or *ote*$^{-/-}$ mutant phenotypes were rescued by increased production of a different LEM-D protein. For these experiments, we generated *dMAN1*$^{-/-}$ and *ote*$^{-/-}$ flies with a single copy of a *P[hsp70:lem-d]* transgene. Four trangenes were generated, with each encoding one nuclear lamina LEM-D protein (dMAN1, Ote, the Bocks α-isoform or the Bocks β-isoform) under the control of the *heat shock 70* promoter (*hsp70*). For each *lem-d*$^{-/-}$, *P[hsp70:lem-d]* line, animals were subjected to a daily heat treatment beginning at the second-instar larval stage of development and the degree of the tissue-specific rescue was assessed in adult progeny (Figure 6).

Rescue of the wing patterning defect in *dMAN1*$^{-/-}$ mutants was used to assess the ability of individual Bocks isoforms or Ote to compensate for dMAN1 loss. All *dMAN1*$^{-/-}$ adults have wings with thick longitudinal veins, a variable number of anterior cross veins, branching of posterior cross veins, and folds in the blade, a phenotype not shared with the other *lem-d* mutants. As a control, we tested whether wing patterning defects were rescued by heat treatment of *dMAN1*$^{-/-}$, *P[hsp70:dMAN1]* animals. We found that all *dMAN1*$^{-/-}$, *P[hsp70:dMAN1]* adults had a normal wing phenotype, indicating that a functional level of dMAN1 protein was produced (Figure 6A). Low levels of rescue of the wing patterning defects (~15%) were also observed in non-heat-treated *dMAN1*$^{-/-}$, *P[hsp70:dMAN1]* adults, implying that basal expression of the *P[hsp70:dMAN1]* transgene is sufficient for phenotypic rescue. Next, we examined wing phenotypes of heat-treated and *dMAN1*$^{-/-}$, *P[hsp70:ote]*, *dMAN1*$^{-/-}$, *P[hsp70:bocks α]* and *dMAN1*$^{-/-}$, *P[hsp70:bocks β]* adults. In all cases, wing-patterning defects remained, implying that ectopic production of Ote or Bocks cannot rescue dMAN1 loss (Figure 6A). As wing defects in *dMAN1* mutants result from upregulation of BMP signaling (Wagner *et al.* 2006; Pinto *et al.* 2008), we conclude that increased expression of neither Ote nor Bocks can restore this signaling in *dMAN1* mutants.

Rescue of the egg laying defect in *ote*$^{-/-}$ mutants was used to assess the ability of individual Bocks isoforms or dMAN1 to compensate for Ote loss. All *ote*$^{-/-}$ females are sterile due to defects in GSC survival (Jiang *et al.* 2008; Barton *et al.* 2013), a phenotype not shared with the other *lem-d* mutants. As a control, we tested whether egg production was restored by heat treatment of *ote*$^{-/-}$; *P[hsp70:ote]* animals. We found that all *ote*$^{-/-}$; *P[hsp70:ote]* females laid eggs (Figure 6B; Figure S3), a rescue that required heat treatment. Next, we tested whether egg production was restored by heat treatment of other *ote*$^{-/-}$, *P[hsp70:lem-d]* animals. Strikingly, we found that increased production of both Bocks isoforms, but not dMAN1, rescued egg laying (Figure 6B; Figure S3), demonstrating that Ote and Bocks share tissue-specific functions that are distinct from dMAN1.

We extended our *ote*$^{-/-}$ rescue studies to investigate the relationship between egg laying and GSC phenotypes. We reasoned that if Ote made multiple contributions during oogenesis, then increased production of other LEM-D proteins

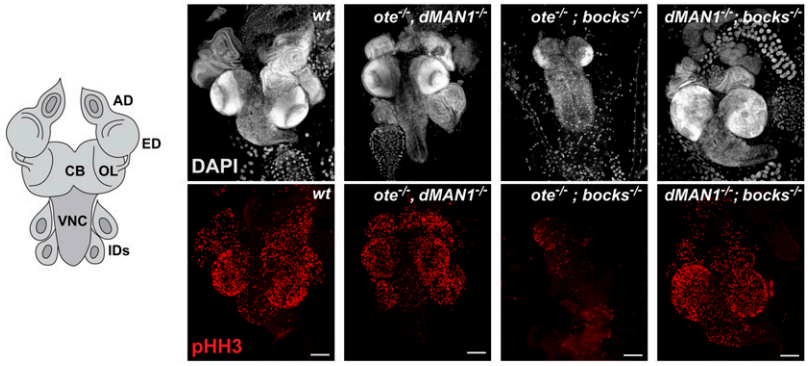

Figure 4 Cellular phenotypes associated with loss of two LEM-D proteins. Left: Schematic of the larval central nervous system and imaginal discs tissues, showing the antennal disc (AD), the eye disc (ED), the optic lobe (OL), the central brain (CB), the ventral nerve cord (VNC), and imaginal discs (IDs). Right: Confocal images of larval tissues stained with DAPI (gray) and antibodies against the mitotic marker phospho-histone H3 (pHH3, red). Tissues were obtained from wild-type (wt) and *lem-d* double mutants (*ote*$^{B279G/PK}$, *dMAN1*$^{\Delta 81/\Delta 26}$; *ote*$^{B279G/PK}$; *bocks* $^{\Delta 10/\Delta 66}$; *dMAN1*$^{\Delta 81/\Delta 26}$; *bocks* $^{\Delta 10/\Delta 66}$) third-instar larvae. Scale bars, 100 μm.

might rescue GSC phenotypes, even though no or few eggs were produced. In *Drosophila* ovaries, GSCs reside within the germarium, a structure comprised of somatic cells niche cells and two to three GSCs (Chen *et al.* 2011; Harris and Ashe 2011; Losick *et al.* 2011). In young *ote*$^{-/-}$ females, ovaries display a complex germarial phenotype, wherein some germaria carry no germ cells, termed GSC loss, and some germaria carry more than five GSC-like cells, termed GSC expansion (Barton *et al.* 2013). To determine the ability of other LEM-D proteins to rescue these phenotypes, ovaries obtained from *ote*$^{-/-}$, *P[hsp70:lem-d]* heat-treated females were stained with antibodies against Vasa, a germline-specific helicase, and Spectrin, a cytoskeletal protein that localizes to the cytoplasmic periphery in somatic cells and forms a spherical structure called the spectrosome in GSCs (Lin *et al.* 1994). As a control for temperature effects, we stained heat-treated *ote*$^{-/-}$ ovaries with Vasa and Spectrin. Surprisingly, these studies revealed that heat treatment alone significantly reduced GSC loss in *ote*$^{-/-}$ ovaries (Figure S4). Even so, differentiation of the *ote*$^{-/-}$ germ cells remained blocked (Figure S4). The reason for the rescue of GSC loss is unknown. In ovaries obtained from heat-treated *ote*$^{-/-}$, *P[hsp70:ote]* females, both GSC loss and germ-cell differentiation were rescued (Figure S3 and Figure S4), consistent with the heat-dependent rescue of egg production.

Next, we examined the germarial phenotypes in ovaries obtained from heat-treated *ote*$^{-/-}$ females carrying the Bocks α-isoform, the Bocks β-isoform, or dMAN1 expression transgene. Increased expression of the Bocks α and Bocks β-isoforms, but not dMAN1, restored GSC numbers and germ-cell differentiation in half of the analyzed germaria (Figure S4), reinforcing the finding that only Bocks is compensating for mutant GSC phenotypes associated with Ote loss. These data imply that the unique requirement for Ote in GSCs reflects insufficient levels of Bocks within the nuclear lamina to buffer against Ote loss. To determine whether Bocks contributes to nuclear lamina function in early stages of germ-cell development, we examined larval gonads. Previous studies have shown that loss of Ote reduces the number of primordial germ cells (PGCs) in the third-instar larval gonads (Barton *et al.* 2013). To understand whether Bocks protects PGCs from Ote loss, we stained *lem-d* double-mutant larval gonads with antibodies against Vasa and Spectrin (Figure 7). We found that *ote*$^{-/-}$; *bocks*$^{-/-}$ gonads had a significantly lower number of PGCs relative to *ote*$^{-/-}$ or other *lem-d* double-mutant gonads (Figure 7B). Further, DAPI staining revealed pyknotic nuclei, indicating that PGC loss is associated with increased cell death in *ote*$^{-/-}$ and in the *ote*$^{-/-}$; *bocks*$^{-/-}$ gonads (Figure 7A insets). Taken together, our data provide strong evidence that Ote and Bocks are functional homologs, with both proteins contributing to germ-cell survival during development.

Discussion

Metazoan genomes encode multiple LEM-D proteins that show enriched localization within the nuclear lamina (Lee and Wilson 2004; Berk *et al.* 2013). *Drosophila* has three nuclear lamina LEM-D proteins, corresponding to dMAN1, Ote, and Bocks. Genetic studies demonstrated that neither *dMAN1* nor *ote* are essential genes, with mutations in these genes causing nonoverlapping tissue-restricted developmental defects, some of which worsen with age (Jiang *et al.* 2008; Pinto *et al.* 2008; Wagner *et al.* 2010; Barton *et al.* 2013). Here, we investigated the developmental requirement of the remaining nuclear lamina *lem-d* gene, *bocks*. We demonstrate that *bocks* is a nonessential gene, with *bocks*$^{-/-}$ adults showing normal morphology, viability, and fertility (Figure 2; data not shown). The absence of mutant phenotypes in *bocks*$^{-/-}$ adults was surprising, given that expression of *bocks* is highest of all *lem-d* genes during development (Chintapalli *et al.* 2007) and that *bocks* is the only *lem-d* gene that displays alternative splicing (Wagner *et al.* 2004). Our data imply that the developmental functions of the nuclear lamina can be maintained, even when total levels of LEM-D proteins are significantly reduced.

Shared LEM-D protein functions extend beyond BAF regulation

We investigated the extent of such regulatory compensation among the three *Drosophila* nuclear lamina LEM-D proteins, using genetic and phenotypic analyses of *lem-d* double mutants. Our studies revealed that loss of any two LEM-D proteins causes lethality before adulthood (Figure 3). These data show that functional redundancy exists among the *Drosophila* LEM-D proteins, as seen for LEM-D proteins in other

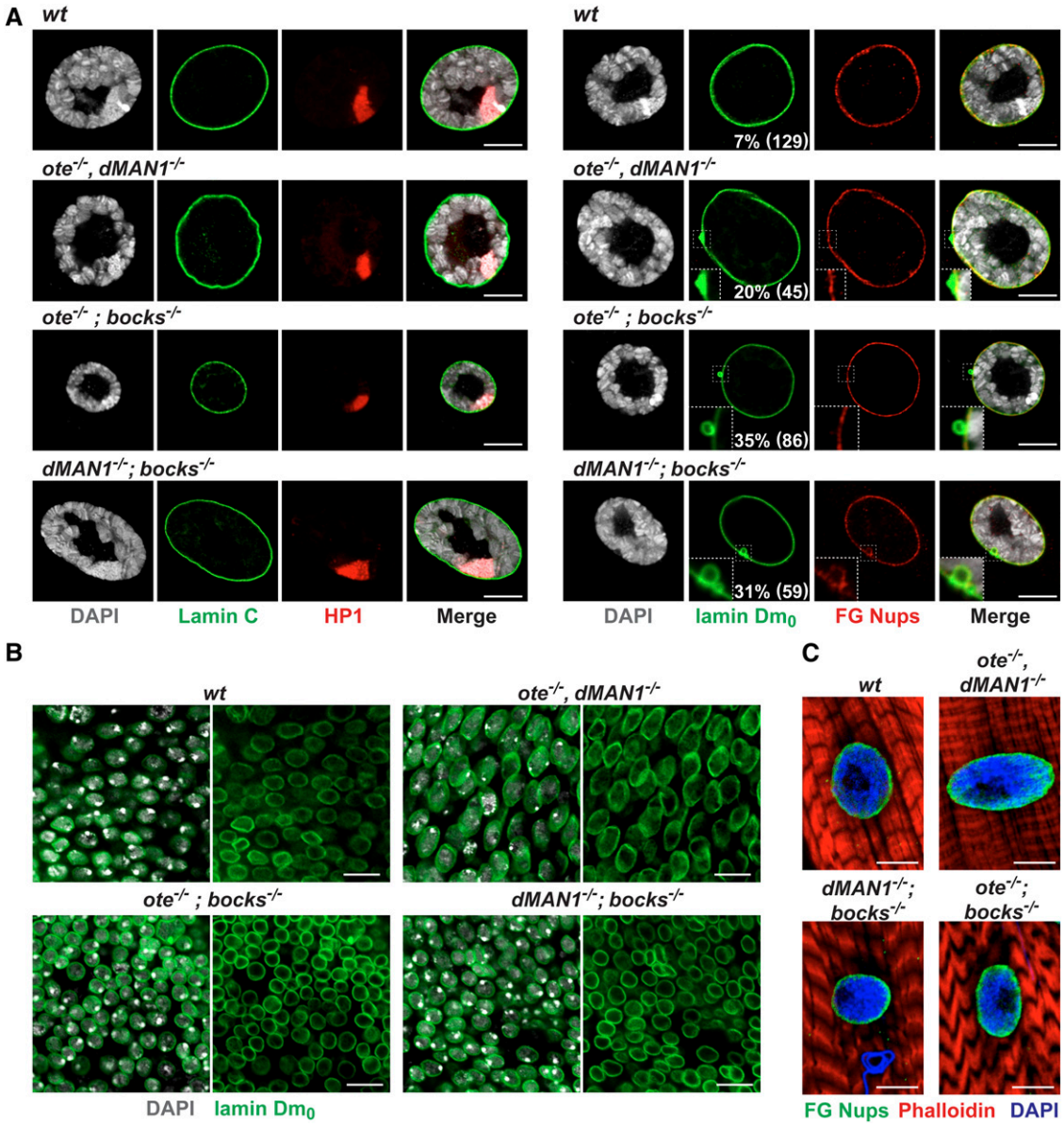

Figure 5 The nuclear lamina remains intact in *lem-d* double mutants. (A) Shown are confocal images of wild-type (wt) and *lem-d* double mutant nuclei of third-instar larval salivary glands. Nuclei were stained for DNA (DAPI, gray), Lamin C, or lamin Dm_0 (green), HP1 (red), or FG-Nups (red). The lamin Dm_0 staining of salivary glands isolated from $bocks^{-/-}$ double mutants showed O-shaped lamin Dm_0 deformities, while the wild-type and $ote^{-/-}$, $dMAN1^{-/-}$ nuclei have solid aggregates of lamin Dm_0 (boxed regions). Numbers in the bottom right corner indicate the prevalence of these deformities. The number of nuclei analyzed from a minimum of five animals is in parentheses. (B) Confocal images of diploid imaginal disc tissues isolated from wild-type (wt) and *lem-d* double-mutant larvae, stained for lamin Dm_0 (green) and DAPI (gray). (C) Confocal images of third-instar larvae body wall muscle nuclei isolated from wt and *lem-d* double mutants. Muscles were stained with DAPI (blue), antibodies against FG-Nups (green), and the F-actin stain phalloidin (red). Genotypes are noted above all image sets. Scale bars, 10 μm.

organisms (Gruenbaum *et al.* 2002; Liu *et al.* 2003; Huber *et al.* 2009; Barkan *et al.* 2012; Gonzalez *et al.* 2012; Reil and Dabauvalle 2013). Interestingly, these studies revealed that although loss of Bocks alone did not affect development, Bocks has multiple roles within the nuclear lamina. We identified dominant genetic interactions only between *bocks* and the other *lem-d* mutants. For example, $ote^{+/-}$; $bocks^{+/-}$ and $dMAN1^{+/-}$; $bocks^{+/-}$ double heterozygous adults showed reduced viability, while $ote^{+/-}$, $dMAN1^{+/-}$ double heterozygous adults did not. Further, no $dMAN1^{-/-}$; $bocks^{+/-}$ adults survived, whereas $dMAN1^{-/-}$, $ote^{+/-}$ adults survived at wild-type levels (Figure S2). The decreased survival of both *dMAN1* and *ote* mutants when Bocks levels are reduced suggests that Bocks compensates for the loss of both proteins. The absence of dominant interactions between *dMAN1* and *ote* mutants suggests that Bocks compensates for different regulatory functions lost in the *ote* and *dMAN1* mutants. The dominant interactions observed with $bocks^{-/-}$

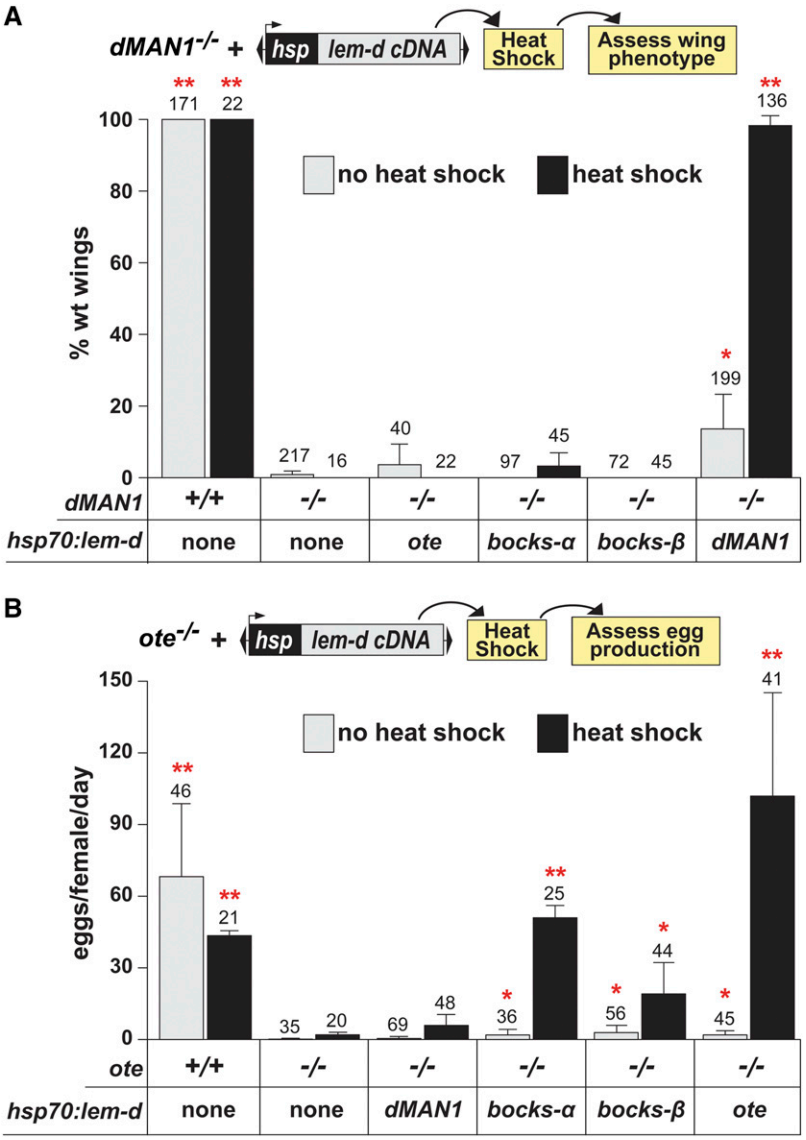

Figure 6 Expression of *bocks* rescues the ovary requirement for Otefin. (A) Top: Schematic of the strategy to assess rescue of the wing-patterning defect in *dMAN1*$^{-/-}$ mutants. Daily heat shocks were given to *dMAN1*$^{-/-}$, *P[hsp70:lem-d]* beginning at second-instar larvae and continued until eclosion. Wing phenotypes of adults were assessed. Bottom: Quantification of the percentage of wild type (wt) wing patterning in non-heat-shocked (gray bars) and heat shocked (black bars) adults carrying either no (none) or the indicated *P[hsp70:lem-d]* transgene. (B) Top: Schematic of the strategy to assess rescue of the egg-production defect in *ote*$^{-/-}$ mutants. Daily heat shocks were given to *ote*$^{-/-}$, *P[hsp70:lem-d]* beginning at second-instar larvae and continued to eclosion. Bottom: Quantification of eggs produced per female per day by wild type and *ote*$^{-/-}$ mutants that were not heat shocked (gray bars) or heat shocked (black bars). The number of adults analyzed is indicated above each bar. Error bars represent standard deviation from a minimum of three independent experiments. Student's *t*-test *P*-values were obtained by comparing data obtained from comparisons between adults of the indicated genotype to *dMAN1*$^{-/-}$ (A) or *ote*$^{-/-}$ (B) adults raised under the same conditions. (* $P < 0.05$, ** $P < 0.01$).

mutants suggests that Bocks provides the strongest compensation for loss of other LEM-D proteins, which may be linked to the higher levels of Bocks in the nuclear lamina (Chintapalli *et al.* 2007).

The hallmark feature of the LEM-D protein family is BAF binding. For this reason, we postulated that the lethality caused by loss of two LEM-D proteins was linked to changes in BAF function. Loss of BAF causes a typical mitotic mutant phenotype (Furukawa *et al.* 2003). Lethality of *baf* mutants occurs at the larval–pupal transition, with larvae carrying small brains and no imaginal discs. These defects are linked to an altered nuclear lamina. Our studies uncovered striking differences between *baf* and *lem-d* double mutants. First, *ote*$^{-/-}$, *dMAN1*$^{-/-}$ and *dMAN1*$^{-/-}$; *bocks*$^{-/-}$ larvae had large brains and imaginal discs and showed high levels of mitosis (Figure 4A). Second, although *ote*$^{-/-}$; *bocks*$^{-/-}$ tissues showed low levels of mitosis as found in *baf*$^{-/-}$ tissues, the *ote*$^{-/-}$; *bocks*$^{-/-}$ mutant phenotypes are different because these mutants die earlier and have nuclei with a normal nuclear lamina structure (Figure 4A and Figure 5). Taken together, these observations suggest that some BAF function is retained in animals with only a single nuclear lamina LEM-D protein and predict that lethality found in the *lem-d* double mutants extends beyond loss of BAF regulation.

How the nuclear lamina LEM-D proteins compensate for loss of a different family member remains unclear. Outside of the LEM-D, none of these proteins show extensive homology (Figure 1B). Nonetheless, all three proteins carry small regions of 8–12 amino acids that show homology to each other (data not shown). Further experimentation is needed to establish the role of these regions in the function of the LEM-D proteins.

Bocks and Ote are functional homologs

As a second approach toward understanding the extent of regulatory compensation among the *Drosophila* nuclear lamina proteins, we determined whether increased expression

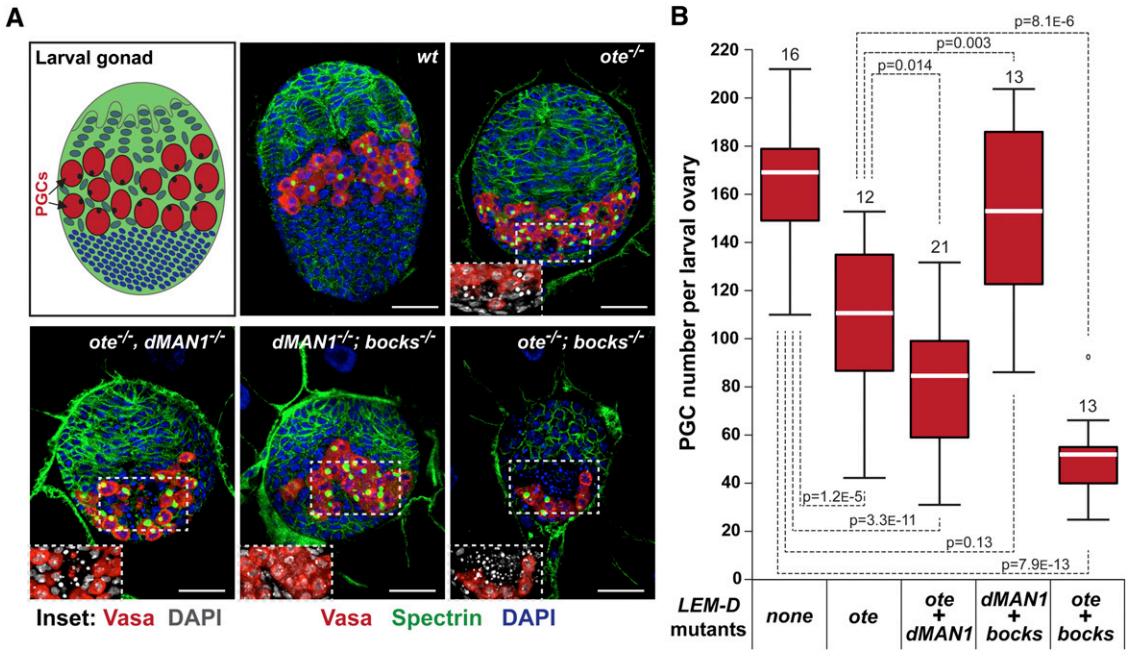

Figure 7 Bocks contributes to survival of primordial germ cells in the developing gonad. (A) Top left shows a schematic of a third-instar larval female gonad, indicating somatic cells (green and blue) and primordial germ cells (PGCs, red). Rest of A shows larval female gonads obtained from wild-type (wt) and *lem-d* double mutants stained for DNA (DAPI, blue), the germline specific helicase Vasa (red) and the cytoskeletal protein Spectrin (green). Boxed areas are magnified below to show pyknotic nuclei indicative of apoptosis. Genotypes are noted above each larval ovary image. Scale bars, 25 μm. (B) Box plots of the number of PGCs per *lem-d* wild-type (none) and double-mutant gonad, with the total number of gonads analyzed for each genotype is indicated above each box plot. For each box, the white line indicates the median PCG number, while boxes and whiskers represent the 25th to 75th percentile interval and the nonoutlier range, respectively. Student's *t*-tests compared differences in PGC numbers between *lem-d* double mutants and controls, with *P*-values between compared pairs shown.

of one LEM-D protein rescued the mutant phenotypes associated with loss of a different LEM-D family member. These experiments revealed that increased amounts of Bocks, but not dMAN1, rescued $ote^{-/-}$ phenotypes, whereas neither Bocks nor Ote could rescue a $dMAN1^{-/-}$ phenotype (Figure 6). We draw several conclusions from these studies. First, we infer that levels of Bocks are limiting in GSCs, thereby sensitizing these cells to Ote loss. Second, we surmise that the Ote function in GSCs is not linked to BAF regulation, because increased dMAN1 expression failed to rescue Ote loss. Third, we infer that dMAN1 contributes regulatory functions distinct from other LEM-D family members, because wing-patterning defects were not rescued by increased expression of Ote or Bocks. Based on these experiments, we suggest that Bocks and Ote are functional homologs. These findings imply that the *Drosophila* nuclear lamina LEM-D family is composed of one MAN1 and two emerin homologs.

We were surprised to discover that the Bocks β-isoform provided partial rescue of $ote^{-/-}$ fertility (Figure 6B). Previous studies have shown that Ote function in GSCs requires the C-terminal peripheral localization domain (Jiang *et al.* 2008), indicating a requirement for nuclear lamina localization. Yet, the Bocks β-isoform lacks the transmembrane domain required for nuclear envelope targeting, which should be incompatible with rescue of $ote^{-/-}$ phenotypes. However, as the Bocks β-isoform directly interacts with lamins (Pinto *et al.* 2008), we reason that these protein associations may be sufficient to target this protein to the nuclear lamina for the rescue of the $ote^{-/-}$ phenotypes.

Multiple factors contribute to tissue-specific defects caused by loss of LEM-D proteins

Loss of LEM-D proteins cause several age-enhanced, tissue-restricted human diseases (Worman *et al.* 2010). Our genetic analyses provide insight into mechanisms that may limit effects of individual LEM-D protein loss to specific tissues. First, our studies support previous findings that LEM-D proteins possess overlapping developmental functions. Importantly, our results suggest that these shared requirements extend beyond BAF regulation. These findings imply that functional motifs outside of the LEM-D share regulatory interactions, even though these regions display limited sequence homology. Second, we find that some LEM-D proteins may possess cell-type-specific functions that are not redundant with other family members. Third, we uncovered evidence that certain cell types may express limiting levels of individual LEM-D proteins, which sensitize the cell toward loss of other LEM-D family members. Taken together, our studies indicate that multiple factors contribute to the restricted nature of LEM-D diseases.

Acknowledgments

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Unique and Shared Functions of Nuclear Lamina LEM Domain Proteins in *Drosophila*

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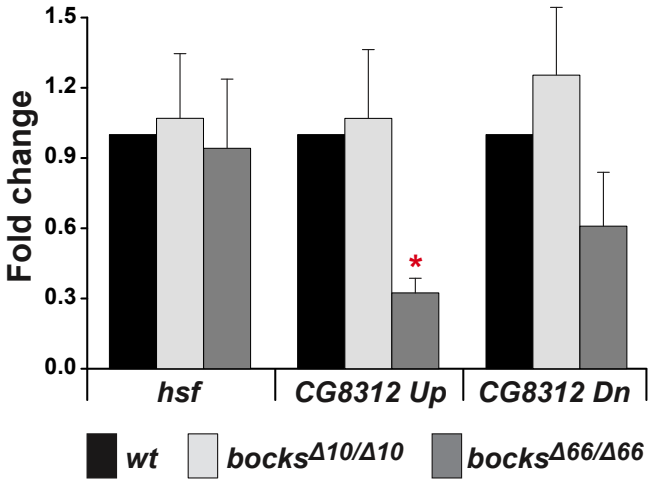

Figure S1 Expression of *CG8312* in *bocks* deletion mutants. Shown is a graph of quantitative real time PCR analyses of RNAs obtained from *bocks*$^{+/+}$, *bocks*$^{\Delta 10/\Delta 10}$ or *bocks*$^{\Delta 66/\Delta 66}$ late, wandering third instar larvae. Fold change is set relative to the value obtained in *bocks*$^{+/+}$ RNA and genes were normalized to *Rpl32,* with expression of *heat shock factor (hsf)* measured as an additional control. Error bars indicate standard deviation from three biological replicates. (*, $p<0.05$, Student's t-test).

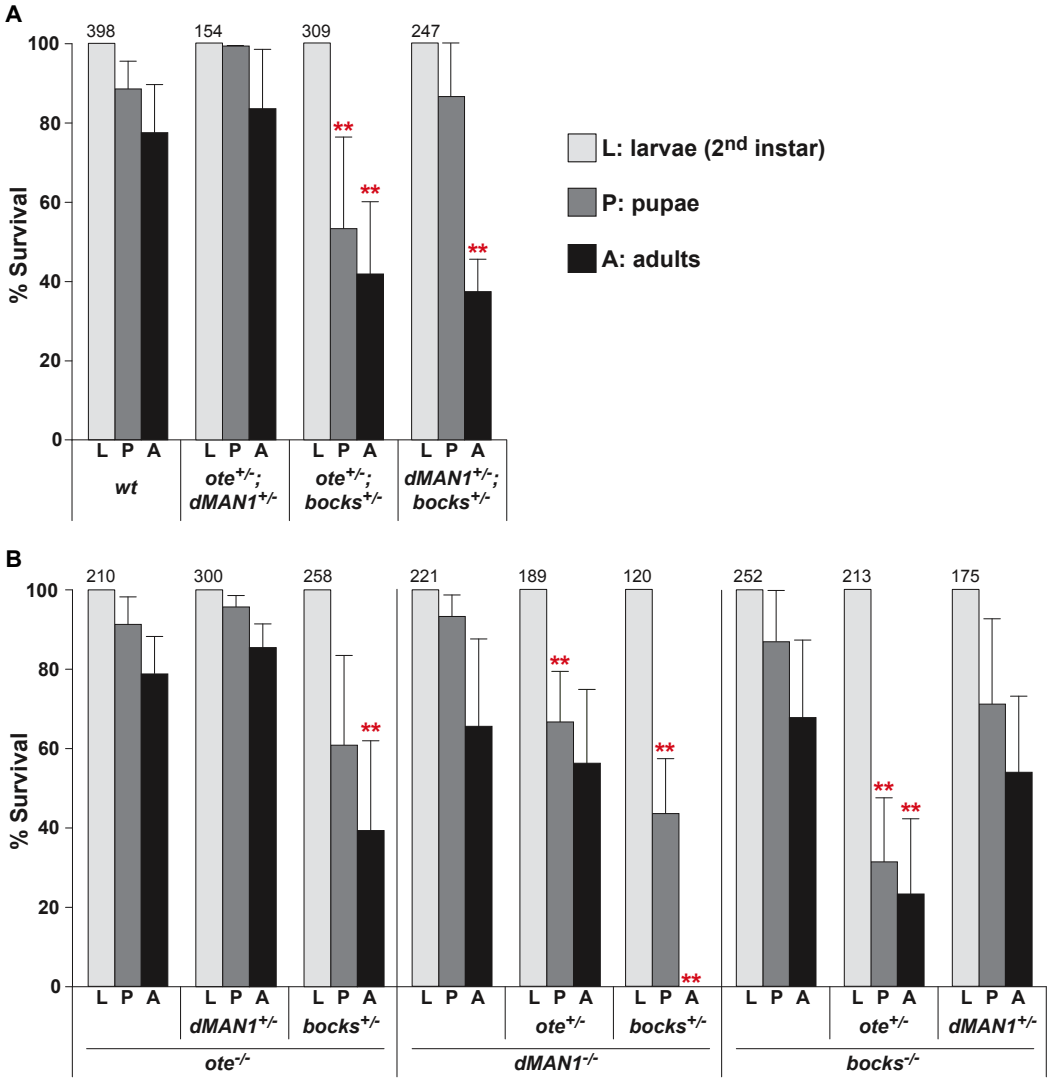

Figure S2 Lethal phase analysis of *lem-d* mutants. **A.** Shown is a graph of the percent survival of collected heterozygous *lem-d* double mutant second instar larvae (L) to pupae (P) and adults (A) of the indicated genotypes. **B**. Shown is a graph of the percent survival of heterozygous and homozygous *lem-d* double mutants, divided into sections by which *lem-d* gene is homozygous mutant. For each graph, the total number of second instar larvae analyzed is listed above each set of bars. At least three independent experiments were completed, with error bars corresponding to standard deviation. Student's t-test p-values were obtained by comparing the percent survival of the heterozygous *lem-d* double mutant with the homozygous *lem-d* single mutant at matching developmental stages (* = $p<0.05$, ** = $p<0.01$).

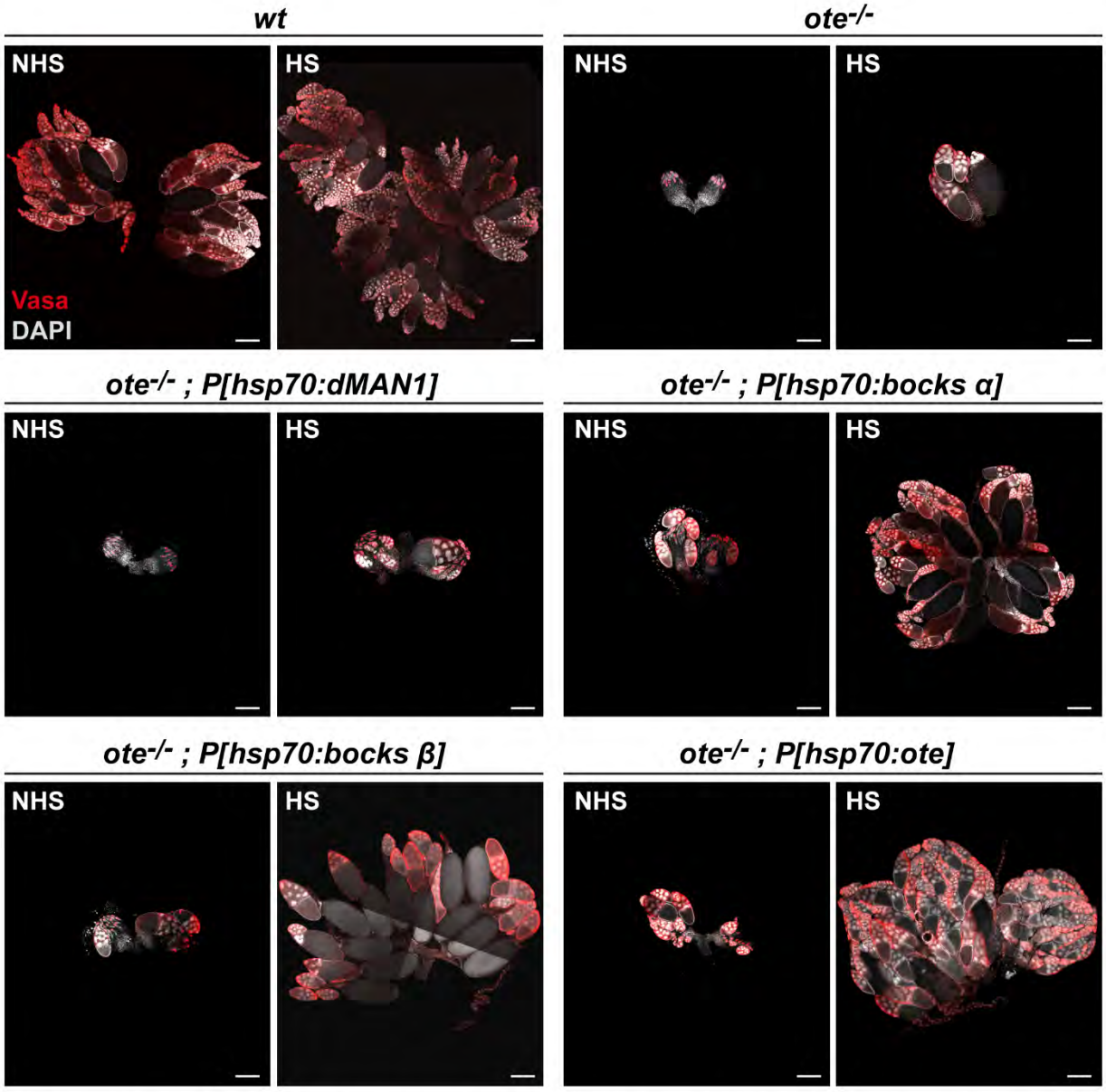

Figure S3 Phenotypes of ovaries isolated from *ote*$^{-/-}$ and *ote*$^{-/-}$, *P[hsp70:lemd]* females. Confocal images of ovaries isolated from two-day-old females stained for Vasa (red) and DAPI (gray). Genotypes are noted above each set of panels that include ovaries isolated from non-heat shocked (NHS) and heat shocked (HS) females. All scale bars represent 100 µm.

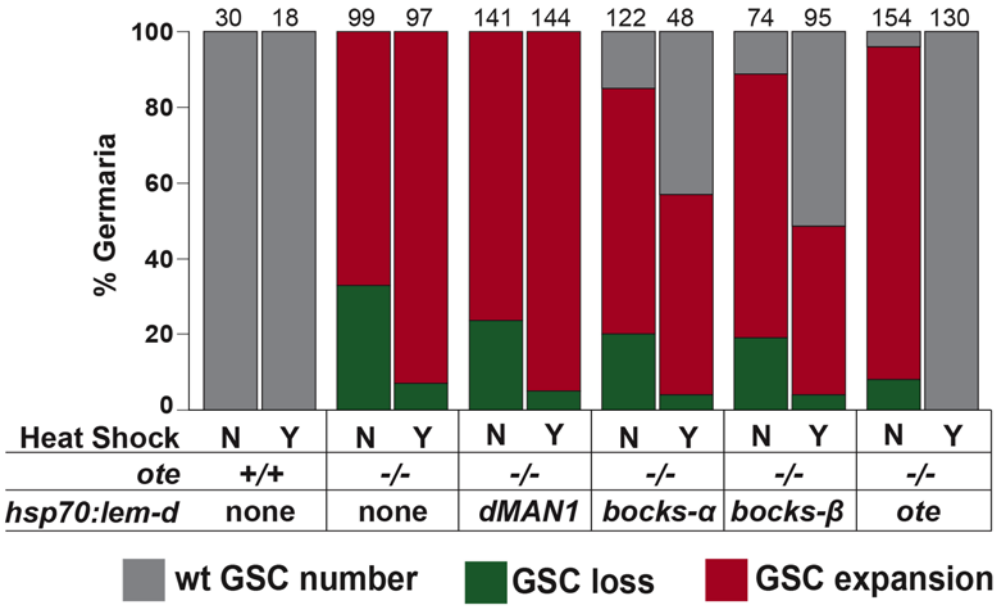

Figure S4 Analysis of germarial phenotypes in *ote*$^{-/-}$ and *ote*$^{-/-}$, *P[hsp70:lemd]* ovaries. Shown is a graph of quantified germarial phenotypes in ovaries obtained from two-day old females that did not receive (N) or received (Y) heat shock treatments during development. Wild type (wt) GSC number (gray) corresponds to germaria with one to three GSCs adjacent to the niche and differentiating germ cells. GSC loss (green) corresponds to germaria that have a complete absence of GSCs or germ cells in the germaria. GSC expansion (red) corresponds to germaria with more than five GSC-like cells and the absence of differentiating germ cells. The ovary genotypes are shown below each pair of bars. The number of germaria analyzed for each genotype and treatment is indicated above the bars. Data were obtained from at least two independent experiments.