Drosophila lysyl oxidases Dmloxl-1 and Dmloxl-2 are differentially expressed and the active DmLOXL-1 influences gene expression and development

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Running title: Drosophila lysyl oxidases Dmloxl-1 and Dmloxl-2

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Mammalian lysyl oxidase (LOX) is essential for the catalysis of lysyl-derived cross-links in fibrillar collagens and elastin in the extracellular matrix, and has also been implicated in cell motility, differentiation and tumor cell invasion. The active LOX has been shown to translocate to the nuclei of smooth muscle cells and regulate chromatin structure and transcription. It is difficult to interpret the role of the LOX protein as it is co-expressed with other members of the LOX amine oxidase family in most mammalian cells. To investigate the function of the LOX proteins, we have characterized the Drosophila lysyl oxidases Dmloxl-1 and Dmloxl-2. We present the gene, domain structure and expression pattern of Dmloxl-1 and Dmloxl-2 during development. In early development, only Dmloxl-1 was expressed, that allowed functional studies. We have expressed Dmloxl-1 in S2 cells and determined that it is a catalytically active enzyme, inhibited by BAPN, a specific LOX-inhibitor. We localized DmLOXL-1 in the nuclei in embryos; in adult salivary gland cells in the nuclei, cytoplasm and cell surface, using immunostaining and DmLOXL-1 antibody. To address the biological function of Dmloxl-1, we raised larvae under BAPN inhibitory conditions and overexpressed Dmloxl-1 in transgenic Drosophila. DmLOXL-1 inhibition resulted in developmental delay and a shift in sex ratio; over-expression in the wm4 variegating strain increased drosopterin production, demonstrating euchromatinization. Our previous data on the transcriptional down-regulation of seven ribosomal and the glue gene under inhibitory conditions and the current results collectively support a nuclear role for Dmloxl-1 in euchromatinization and gene regulation.

Lysyl oxidase (LOX) is an extracellular matrix amine oxidase (1, 2). In addition to its essential role in the catalysis of lysyl-derived cross-links in fibrillar collagens and elastin, LOX has been implicated in cellular processes, including motility, differentiation, ras-transformed reversion and tumor cell invasion. The active extracellular LOX has been shown to translocate to the nuclei of smooth muscle cells (3, 4). Histones H1 and H2 were demonstrated to be substrates of LOX in vitro (5, 6). A previous report confirmed a role for the nuclear LOX in regulating the chromatin structure in antisense LOX-treated ras-transformed NIH 3T3 cells (7).

The LOX protein contains a C-terminal region highly conserved in all mammalian species reported, including the copper-binding site, the lysyl and tyrosyl residues that form the carbonyl cofactor, and a cytokine receptor-like domain. Four novel mammalian lysyl oxidase-like proteins, LOXL (8, 9), LOXL2 (10, 11), LOXL3 (12, 13) and LOXL4 (14, 15) have been described, all of which contain the same conserved C-terminal domains necessary for catalytic activity. LOX, LOXL, and LOXL4 have been reported as active amine oxidases inhibited by BAPN, using collagen, elastin, and synthetic substrates (16, 17, 18). In addition, LOXL2, LOXL3 and LOXL4 have four Group A SRCR domains in their conserved N-terminal region. SRCR domains are evolutionarily conserved with either 6 (Group A) or 8 (Group B) cysteine residues. SRCR domains are
candidates for protein interactions, and although they have been described in a large variety of proteins, including molecules participating in immune signaling processes, their function is mostly unknown (19).

BAPN, a well-known irreversible inhibitor of the LOX enzyme, is routinely used to evaluate the amine oxidase activity of LOX in assays using cell and tissue extracts (2, 20). BAPN has also been extensively used in in vitro and in vivo tests to evaluate the biological role of LOX (21). These studies demonstrated that BAPN inhibition of LOX results in alterations in a range of processes including cell proliferation (22), motility (23, 24), migration (23), embryonic development (25), development (26), differentiation (27), morphogenesis (28), wound healing (29), tumor cell reversion (30, 31, 32) and invasion (33). However, the results of these experiments are increasingly difficult to interpret, since the LOX-like amine oxidases, also inhibited by BAPN, are co-expressed with LOX in many cells and tissues (1, 13, 33, 34, 35).

To address the individual function of LOX, null mice were recently generated. The LOX null mice demonstrate cardiovascular dysfunction, aortic aneurysms, ruptured diaphragms, and die soon after birth (36, 37). These phenotypes confirmed an essential role of LOX, but apart from the consequence of the elastin and collagen cross-linking deficiencies, did not reveal other functions. Several cellular studies were initiated to uncover the intracellular function of LOX in transformation and tumor cell invasion. In these experiments, LOX was inhibited by antisense LOX, and over-expressed as the full length 50 kDa and the active 32 kDa forms (6, 31, 33). The results confirmed significant roles in cell phenotype control, however, the molecular mechanisms influenced by LOX remained largely unclear.

In search of an animal model to study the individual roles of LOXs, we have identified two lysyl oxidase genes, Dmloxl-1 and Dmloxl-2, in Drosophila melanogaster. Unlike the mammalian LOXs, Dmloxl-1 and Dmloxl-2 demonstrate a differential expression pattern. Dmloxl-1 is expressed throughout development and adulthood, while Dmloxl-2 is expressed only in adults. We have also demonstrated that in vivo BAPN inhibition of DmLOXL-1 reduced the steady state mRNA levels of seven ribosomal protein genes (38). These results supported the nuclear role of LOX previously observed in NIH 3T3 cells, and the hypothesis regarding the potential role of LOX in regulating overall chromatin structure, and consequently transcription (7).

Changes in chromatin organization can be monitored in Drosophila using heterochromatin-induced gene silencing in the well-established position effect variegation (PEV) model. In the w^m4 strain, the w gene, responsible for red eye color, is placed close to the heterochromatin boundary as a result of chromosomal inversion. In the variegating eye phenotype of the w^m4 strain, the expanding heterochromatin silences the clonally initiated expression of the w gene in some cells, resulting in more white eye cells. In contrast, euchromatinization results in more red eye cells (39). Changes in heterochromatinization can be monitored by the ratio of red and white eye cells, and quantified by the concentration of drosopeterin (40).

In this study, we describe the structure and expression of the Dmloxl-1 and Dmloxl-2 genes; characterize DmLOXL-1 as a BAPN-inhibited, catalytically active amine oxidase; demonstrate nuclear localization of DmLOXL-1 using immunohistochemistry; test the biological function of DmLOXL-1 during development using in vivo inhibitory conditions and a transgenic Drosophila strain to over-express Dmloxl-1; and monitor changes in chromatin structure induced by increased DmLOXL-1 expression in transgenic flies.

**MATERIALS AND METHODS**

**Dmloxl-1 and Dmloxl-2 cDNAs and chromosomal mapping** - The Berkeley Drosophila Genome Project (BDGP, www.fruitfly.org) expressed sequence tag (EST) database was searched by the TBLASTX program using the conserved C-terminal end of the human LOX cDNA sequence M94054 (GenBank Accession Number M94054) as a query. Two different, but homologous, Drosophila EST sequences GH05569 (GenBank Accession Number BT001394) and LP04931 (GenBank Accession Number A1260913) were identified and fully sequenced using a Perkin-
Elmer ABI Prism 310. For chromosomal localization of the corresponding genes, the cDNA inserts were isolated from the recombinant clones and labeled with digoxigenin, according to the manufacturer's protocol (Boehringer Mannheim). The probes were hybridized overnight to polytene chromosome squashes prepared from third-instar larvae salivary glands of *Drosophila melanogaster* as previously described (41). Bound probe was visualized with antidigoxigenin coupled to horseradish peroxidase (Roche) and diaminobenzidine (DAB, Sigma), to identify the chromosomal location of both genes.

**Primer extension** - The *Dmloxl-1* specific oligonucleotide 5' - AACTTGGCCTGAGAACCACA-3' and the *Dmloxl-2* specific oligonucleotide 5'-CAGGACCTCAATGTTGCCT-3' were radiolabeled at their 5' termini by polynucleotide kinase and [γ-32P]dATP, and used in primer extension experiments to determine the 5' end of the transcripts (42). A cDNA synthesis kit was utilized to prepare extension products as described by the manufacturer (Life Technologies, Inc.). The sizes of the extended DNA products were determined on a 6% sequencing gel by comparison with the nucleotide sequence of pUC plasmid DNA generated by the m13 primer.

**Developmental Northern analysis** - Synchronized cultures of *Drosophila* Canton-S strain were established. Adult heads, adult male bodies and adult female bodies were collected for RNA extraction using RNA-STAT-60 (Tel Test, Inc.) according to the manufacturer's protocol. Aliquots of 10 µg total RNA were electrophoresed in a 1.2% formaldehyde-agarose gel and blotted onto Hybond N+ nylon membrane (Amersham Biosciences). 32P-radiolabeled cDNA inserts of EST clones GH05569 and LP04931, and rp49, a ribosomal standard, were used as probes for Northern analysis.

**PCR expression profiling of Dmloxl-1 and Dmloxl-2** - First strand cDNA representing total mRNA for a given developmental stage in *Drosophila* was obtained from OriGene Technologies, Inc. The amount of template per PCR reaction was 1 ng. The panel of templates was normalized by OriGene for equivalent amounts of rp49 cDNA. PCR primer pairs used were: For *Dmloxl-1* Forward: 5'-GACGCTTACGAGATCCGAGAC-3' Reverse: 5'-TCTTAACCAAGCAGGCTTTTG-3' for *Dmloxl-2* Forward: 5'-GTGTGGCAAGGCGGTATC-3' Reverse: 5'-GCTGCGAATTTCACGACTTCG-3' for *Dmloxl-2*. Standard PCR reactions were prepared; 1.25 units of Taq DNA polymerase (Promega), Taq buffer (Promega), 2.5 mM MgCl2, 80 µM each dNTP, 1.25 pmoles of each primer, and 1 ng template in 25 µl reactions, using 5 min 94°C initial denaturation followed by 35 cycles of 1 min 94°C denaturation, 1 min 60°C annealing, and 1 min 72°C elongation, followed by 5 min 72°C terminal extension. The reaction products were displayed on a 1.25% agarose gels with 0.5 µg/ml EtBr in 1x TAE and photographed.

**Immunohistochemical detection of DmLOXL-1 in embryos and salivary glands** - Fresh 0-6 hrs Canton-S embryos were collected, dechorionated in 50% bleach and fixed in 1:4 dilution of freshly made 4% paraformaldehyde: n-heptane for 20 min (43). The embryos were rehydrated and blocked for 30 min with 10% normal goat serum (Molecular Probes Inc.) and then incubated with 1:1000 Lamin Dm0 antibody and either pre-adsorbed 1:300 DmLOXL-1 antibody or 1:300 preimmune serum at 4°C overnight. The monoclonal lamin Dm0 antibody, ADL67, was obtained from the Developmental Studies Hybridoma Bank, University of Iowa (44, 45, 46). After six washes of PBS with 1% Triton X-100, the samples were incubated with secondary antibodies at 4°C for 1 hour. Secondary antibodies were Alexa Fluor 546 goat anti-mouse IgG and Alexa Fluor 488 goat anti-rabbit IgG (Molecular Probes Inc.) Embryos were mounted on slides, with Vectashield mounting medium for fluorescence with DAPI (H-1200,
Vector Laboratories Inc.), and visualized on Zeiss LSM5 Pascal confocal microscope using x100 oil immersion objective. Polyten chromosome squashes were prepared from third-instar larval salivary glands as previously described (47). DmLOXL-1 antibody and pre-immune serum were used in 1:250 dilutions. RNA polymerase II monoclonal antibody (rabbit, 48) was used in 1:500 dilution. Fluorescein i-thiocyanate-conjugated goat anti-mouse secondary antibody Alexa Fluor 488 (Molecular Probes Inc.) was diluted at 1:300 to detect RNA polymerase II staining, and goat anti-rabbit Cy3-conjugated secondary antibody Alexa Fluor 555 (Molecular Probes Inc.) diluted at 1:300 was used to visualize DmLOXL-1 staining. Fluorescence of the tissue specimens was photographed using appropriate filters.

Activity measurement of recombinant DmLOXL-1 - The coding sequence for Dmloxl-1, using the cDNA clone LP49319 (Research Genetics) was amplified, without the signal sequence, by using primer-adaptors and Deep Vent DNA polymerase (New England BioLabs) in a PCR reaction containing LP4931 cDNA as template. The product was trimmed by appropriate restriction enzymes, then inserted into a vector containing a metallothionein promoter and the coding sequence for BiP’s signal sequence, and fused at the 3’ end to a stop codon linked to a transcription terminator (pMT/BiP/V5-His, Invitrogen Corp). Constructs were amplified in E. coli and inserts were sequence verified.

The expression construct was co-precipitated with a plasmid coding for hygromycin resistance (pHygro, Invitrogen Corporation) using calcium phosphate. Drosophila S2 cells in complete medium were exposed to the precipitate for 24 hours. The cells were moved to fresh complete medium with 500 µg/ml hygromycin, cultured until resistant cells began to grow, and then were progressively transferred into serum free medium (JRH’s Excell-420) containing hygromycin. For expression, recombinant cells were grown to 5 x 10⁶ cells per ml in shake flasks at room temperature, 0.5 mM copper sulfate was added and the cells were cultured for two more days.

Cells were separated from culture medium by low speed centrifugation. The medium was diluted with equal volume of water, 120 g/l urea and 2 ml/l of cibacron blue bound to agarose (CBA) was added, and the suspension was stirred for 60 minutes at room temperature. CBA with bound Dmloxl-1 was centrifuged out of the mixture, resuspended in 2 M urea in tris buffered saline (TBS; 50 tris-HCl, pH 7.6, 140 mM NaCl), transferred to a column, washed with TBS-2M urea and Dmloxl-1, eluted with TBS-6M urea. Copper content of Dmloxl-1 was measured by atomic absorption chromatography. Standards were copper sulfate dissolved in TBS-2M urea, and the same buffer was used for the samples. Five readings of each sample were taken and averaged for comparison to the standard curve. Copper content was determined after removal of extraneous copper from samples by dialysis against EDTA.

Recombinant Dmloxl-1 in TBS-6M urea was dialysed sequentially at 4 °C against TBS-2M urea containing 10 mM 2,2'-dipyridyl for one day, 0.5 mM CuSO₄ for 2-4 days, 10 mM EDTA for one day, and a final dialysis against TBS-2M urea for one day to remove EDTA. Initial rate of amine oxidation was measured as the rate of hydrogen peroxide formation. Amplex Red (Molecular Probes) was used as the reductant and indicator with horseradish peroxidase to measure the production of hydrogen peroxide (49). Fluorescence of oxidized Amplex Red was measured by a Tecan plate reader. One hundred microliters of TBS containing Amplex Red, horseradish peroxidase and substrate were placed in the well of a microplate. Final substrate concentration of recombinant tropoelastin was 0.5 mg/ml. Reactions were started by adding 100 µl TBS containing 0.5 µg Dmloxl-1 for a total concentration of 67.5 nM, with 40 mM urea with or without BAPN at 30 °C. Excitation was at 560 nm and emission read at 590 nm. Readings were taken every 30 seconds for 300 seconds.

DmLOXL-1 inhibition in vivo by BAPN - BAPN was dissolved in standard Drosophila food in a series of concentrations from 0.05 to 10 mM (20). Synchronized 0-1 hour wild-type Canton-S embryos were transferred to the medium and development was followed by microscopic observation of the population.

Dmloxl-1 transgenic flies and development of a homozygous strain - The cDNA insert
of the LP04931 EST containing the full coding region of the Dmloxl-1 gene was excised with EcoRI and XhoI and ligated into the SalI–EcoRI linearized P-element derivative pP[hsneo] vector (50). The construct was injected into white (w) embryos. Male progeny were pairwise crossed with Oregon-R virgins. Emerging larvae were transferred to media containing 10 mM neomycin and kept permanently at 29°C to induce the expression of the construct by the heat shock promoter. All of the Drosophila strains used in these experiments were from the collection of the Department of Genetics and Molecular Biology, University of Szeged, Hungary.

The transgenic virgins were pairwise crossed with a multibalanced strain: pP[hsneoDmloxl-1] × BcGla; TM3GFP Male offspring with Cy;Sb phenotype were collected and crossed with virgins from the same multibalanced strain. The hatched larvae were raised as above. To follow the balancer cross-links, the phenotype of the dead flies was examined daily. The surviving flies were either Gla or Cy.

In order to generate pP[hsneoDmloxl-1]/pP[hsneoDmloxl-1] homozygotes, Cy or Gla flies from the above cross, that survived the neomycin selection, were pairwise crossed, and homozygotes emerged without second chromosomal balancer. The viability of homozygous transgenic flies, and the loss of the second chromosome balancer indicated that the integration had not occurred within an essential locus.

Real-time PCR analysis of Dmloxl-1 expression in Dmloxl-1/Dmloxl-1 transgenic Drosophila - Adult males and females of Dmloxl-1/Dmloxl-1 transgenic, heat-shocked (29°C) non-transgenic, and heat-shocked Dmloxl-1/Dmloxl-1 transgenic animals were collected for RNA extraction using RNA-STAT-60 (Tel Test, Inc.) according to the manufacturer’s protocol. After DNase treatment, 1 µg of RNA was used for cDNA synthesis, using Superscript II (Gibco) with random hexamer primers. RNAse treatment (37°C, 20 min) followed the first strand synthesis. The real time PCR was performed using the DNA Engine Opticon 2 system (MJ Research, Inc.). Dmloxl-1 forward primer: 5’-GCTGTATGGACAGTGATGCCTC-3’ reverse primer: CCAGTGTAGCTAAAGAGGC, and rp49 control forward primer: 5’- TCCATACCAGTTCAAGATCAG-3’ and reverse primer: 5’- GTGTATTCCGACCAGTTAC-3’. Standard reactions were done in triplicates, in 25 µl volumes, using 1 µl cDNA, and DYNamo master mix (Finzymes Oy) containing an optimized PCR buffer with Tbr DNA polymerase, SYBR Green I fluorescent dye, 5 mM MgCl2, and dNTP. Initial denaturation occurred at 95°C for 5 min, and each cycle consisted of a 95°C 10 sec denaturation, 60°C 30 sec annealing, and 72°C 30 sec elongation. Fluorescence was measured at the end of every extension step, at 83°C, for 39 cycles. Product specific amplification was confirmed by melting curve analysis at the end of the thermocycle profile. The threshold cycle line was established by Opticon Monitor 2 software (MJ Research, Inc.) at the linear portion of the log scale curve, and the ratio of Dmloxl-1 to rp49 was calculated using the 2-^∆∆CT method (51). Melting curves were obtained at a range from 65°C to 92°C at 0.2°C steps to ensure the quality of the reactions. Statistical significance was calculated using Unpaired t-test.

Drosopterin quantitation - The red eye pigment drosopterin was extracted as described previously (40) from 15 heads of anesthetized wild type, white eye control (w), variegating eye control (w m4; CyRoi/Sco) strains without the transgene, variegating male (w m4/Y; Dmloxl-1/Dmloxl-1) and variegating female lines w m4/w m4; Dmloxl-1/Dmloxl-1 with two copies of the transgene. The Oregon-R strain was used as the red eye control. Transgenic females and males were derived from the same progeny. Drosopterin concentration was measured using spectrophotometer as previously described (40).

RESULTS

The Drosophila melanogaster lysyl oxidase-like 1 (Dmloxl-1) gene

Sequencing of the EST clone LP04931 identified a partial cDNA that demonstrated homology to the human lysyl oxidases and was
named *Drosophila melanogaster* lysyl oxidase like-1 or *Dmloxl-1*. Using this partial cDNA sequence as a query, BLASTN searches of the *Drosophila* Genome Project genomic database identified overlapping BAC clones BACR10009 (GenBank Accession Number AC009349) and BACR02G16 (GenBank Accession Number AC008287) that contained the full *Dmloxl-1* gene. Both BAC clones had been mapped to the 100C4 band of chromosome 3. *In situ* hybridization using LP04931 cDNA as a probe verified that the *Dmloxl-1* gene maps close to the tip of the right arm of the third chromosome, within the 100C band (Fig. 1A). The 5' end of the *Dmloxl-1* transcript was established by primer extension, that added 142 nucleotides to the LP04931 EST sequence (Fig. 1B). A consensus TATA motif is located 31 nucleotides upstream from the putative transcription start site. An in-frame stop codon (TAA) precedes the putative translational start codon ATG, and a potential polyadenylation site (AATAAA) follows the translational stop codon (TGA). This open reading frame yields a putative peptide sequence of 360 amino acids with a predicted molecular mass of approximately 40 kDa.

**The Drosophila melanogaster lysyl oxidase-like 2 (Dmloxl-2) gene**

The GH05569 cDNA clone was identified as a partial cDNA that was distinct from *Dmloxl-1*. Two overlapping genomic clones BACR13F14 (GenBank Accession Number AC007803) and BACR07G11 (GenBank Accession Number AC007802) containing the *Dmloxl-2* cDNA were identified by further BLASTN searches of the *Drosophila* genomic database. Both clones were mapped to the 58A1-2 band of chromosome 2. *In situ* hybridization using cDNA GH05569 as a probe verified that the *Dmloxl-2* gene maps to the 58A band of the second chromosome (Fig. 1A). The 5' end of this cDNA was established by primer extension that produced two equimolar signals (Fig. 1B). Two TATA-like sequences were identified, one (AATAT) 47 bp upstream from the start site of the longer transcript, and the other (AAATAT) 32 bp upstream from the start of the shorter transcript. A possible polyadenylation signal (ATTAAA) followed the translational stop codon (TGA). This open reading frame yields a putative peptide sequence of 511 amino acids with a predicted molecular mass of 58 kDa.

Comparison of the full-length cDNA sequence to genomic DNA allowed identification of intron sequences. The *Dmloxl-1* gene contains a single 55 bp intron within the 3' region. The *Dmloxl-2* gene contains three introns, the third also 55 bp long, and spaced at the same conserved site as in *Dmloxl-1* (Fig. 1D), indicating a potential gene duplication event. The copper-binding domain of *Dmloxl-2* is split by an intron (second intron) at the same position as in all human LOX and LOX-like genes (Fig. 2).

Extensive BLAST searches of the *Drosophila* genome and annotated proteome using *Dmloxl-1* (GenBank Accession Number AJ295625) and *Dmloxl-2* (GenBank Accession Number AJ295626) sequences did not result in the identification of any other potential lysyl oxidase gene.

**Domain structure comparison**

The predicted amino acid sequences of DmLOXL-1 and DmLOXL-2 both contain an N-terminal signal peptide and a LOX-like domain (Fig. 2) that is characterized by a Cu²⁺-binding site with spatially conserved four histidines, the lysyl and tyrosyl residues that form the unique quinone cofactor, and a cytokine receptor-like domain (1). DmLOXL-1 contains one N-terminal SRCR domain, and DmLOXL-2 contains two N-terminal SRCR domains. Mammalian lysyl oxidases contain either none: LOX (2), LOXL (8) or four SRCR domains: LOXL2 (10), LOXL3 (12, 13) and LOXL4 (14, 15). Both *Drosophila* proteins have predicted nuclear localization sequences and predicted signal sequences, as detected by PSORT (http://psort.nibb.ac.jp/).

The sequence of the most conserved 122 amino acid region that contains the copper-binding site and the cytokine receptor-like domain of DmLOXL-1 shows high homology to the human lysyl oxidases: to LOX 48%, LOXL 50%, LOXL2 48%, LOXL3 49%, and LOXL4 53%. The same region of DmLOXL-2 shows homology to LOX 45%, LOXL 48%, LOXL2 53%, LOXL3 51%, and to LOXL4 48%.
Differential expression of Dmloxl-1 and Dmloxl-2 during development

Dmloxl-1 is expressed as a 1.5 kb mRNA, and Dmloxl-2 as two mRNAs of 1.7 and 1.8 kb (38). These sizes are consistent with the sizes of the predicted DNA sequences and the results of the primer extension experiments (Fig. 1B). Our earlier results demonstrated expression of Dmloxl-1 in five developmental stages tested, including 0-8 and 8-16 hour embryos, 3rd instar larvae, 48 hour pupae, and adult flies. Expression of Dmloxl-2 was present only in adults (38). Northern blot analysis of adult heads, male and female bodies detected abundant Dmloxl-1 mRNA in the heads and male bodies and much less in female bodies (Fig. 1C). To further characterize Dmloxl-1 and Dmloxl-2 expression during development, quantitative PCR was performed using RNA from 12 different stages and four adult body parts. Dmloxl-1 mRNA was found predominantly in pupae, at lower levels in 4-12 h embryos and 3rd instar larvae; and even lower in 1st and 2nd instar larvae. Female adults expressed Dmloxl-1 mRNA at very low levels (results not shown).

Nuclear localization of DmLOXL-1 in embryos and adult salivary gland cells

Previous nuclear localization data for LOX (4, 7), and LOXL (52), and the presence of the nuclear localization signal within DmLOXL-1 prompted us to test if DmLOXL-1 was present in the nucleus. We performed immunostaining of 0-6 hrs embryos, using DmLOXL-1 and lamin Dm0 antibodies, and of adult salivary glands and polytene chromosome squashes using DmLOXL-1 and RNA polymerase II antibodies. As expected, lamin Dm0 antibody stained the nuclear lamina (Fig. 3A) and we observed intense punctate staining of DmLOXL-1 within the nuclei of these embryonic cells (Fig. 3B). In adult salivary gland squashes, RNA polymerase II antibody strongly stained the polytene chromosomes (Fig. 3C, D). There was some punctate DmLOXL-1 staining on these chromosomes, but no distinct pattern could be observed (Fig. 3E). Within the nuclei of adult salivary gland cells, nuclear DmLOXL-1 staining was present, but the staining intensity was reduced compared to embryonic cells. In these cells, cytoplasmic and pericellular DmLOXL-1 was also present (Fig. 3E). Preimmune serum did not react with either nuclei of embryos (not shown), salivary gland cells or with polytene chromosomes (Fig 3F).

DmLOXL-1 catalytic activity

The activity of the recombinant DmLOXL-1 was tested using purified recombinant tropoelastin substrate. DmLOXL-1 was inhibited by BAPN in the presence of a saturating concentration of tropoelastin-derived peptidyl lysine (Fig. 4). Fifty percent inhibition, derived from the dose response curve, required a 5.5 molar excess of BAPN over recombinant DmLOXL-1. One hundred percent inhibition was not observed at higher concentrations of BAPN.

DmLOXL-1 inhibition results in a developmental delay and an altered sex ratio

The distinctly different temporal expression patterns of the two Drosophila lysyl oxidase genes provided a unique model to study DmLOXL-1 as the only active lysyl oxidase in developing embryos, larvae and pupae. To study the function of the DmLOXL-1 during development, we utilized BAPN inhibition. There are no previous Drosophila studies using BAPN in vivo. Furthermore, as the concentration of BAPN in the food does not equal the concentration within tissues, and there is no established method to measure DmLOXL-1 activity in Drosophila tissue samples, we raised Drosophila on food containing a range of BAPN concentrations. Canton-S flies grown on BAPN supplemented media exhibited a concentration-dependent developmental delay. Drosophila raised in medium containing 5 mM BAPN emerged from pupation 30 hours later than controls with reduced viability, while lower range of the BAPN concentration series (0.05 mM – 1 mM) did not delay development. Food containing 10 mM BAPN drastically reduced viability and increased the normal 11-day development to 20-25 days with long larval stages and reduced larval locomotion. In subsequent phenotypic characterization experiments, we used 5 mM BAPN, as this concentration was sufficient to cause an effect without a major reduction in viability.
In addition to the 30-hour developmental delay, we observed a shift in the male-female ratio in the BAPN treated animals. In six independent BAPN feeding experiments in a total population of 1743, the mean relative percentages of males and females were 38.2% and 61.8%. In the control population of 1124 individuals, the male and female percentages were 48.8% and 51.2% (Fig. 5). This observation demonstrated that males were more sensitive to the BAPN-induced decrease of active DmLOXL-1. The flies raised on 5 mM BAPN were fertile as adults. Furthermore, the DmLOXL-1-inhibited adult males demonstrated, compared to females, a decreased number of red eye cells in w^m4 strain (data not shown).

**Dmloxl-1 over-expression suppresses position-effect variegation**

To evaluate the effect of Dmloxl-1 over-expression, we generated pP[hsneoDmloxl-1]/pP[hsneoDmloxl-1] homozygous transgenic line. Integration of the transgene was mapped by balancer loss to the second chromosome. The increased expression of the Dmloxl-1 mRNA in transgenic animals was confirmed by real-time RT-PCR following heat induction, using total mRNA from 15 flies for each group, normalized to rp49 mRNA levels. Non-transgenic females showed a significant, 114% increase (p < 0.05) in Dmloxl-1 levels following heat induction, while levels in induced males did not increase. In transgenic females, Dmloxl-1 mRNA showed a significant, 80% increase (p < 0.025) over the Dmloxl-1 mRNA expression in heat-shocked non-transgenic females, and 28% increase in heat-shocked males over heat shocked control males (Fig. 6).

We tested the effect of increased Dmloxl-1 gene dosage (two autosomal and two transgenic copies) in homozygote transgenic animals using the w^m4 allele of the variegating locus white-mottled. Suppression of white variegation (more red eye color) was observed in Dmloxl-1/Dmloxl-1 homozygotes in both sexes. The amount of the red eye pigment, drosoprotein, significantly increased in both male (12.3 +/- 0.43) and female (25.4 +/- 0.98) transgenic animals compared to control variegating (w^m4; CyRoi/Sco) strain (7.53 +/- 0.27) indicating a more open chromatin structure in the w^m4 animals. Females demonstrated significantly (p < 0.0001) higher levels of drosoprotein than males (Table 1).

**DISCUSSION**

In contrast to the five mammalian lysyl oxidases, in *Drosophila* there are only two lysyl oxidase-like proteins, DmLOXL-1 and DmLOXL-2. The two Dmloxl genes are localized on chromosomes 3 and 2, respectively. The single intron in Dmloxl-1 and the third intron in Dmloxl-2 are conserved: they are the same length, 55 base pairs, and have the same splice site. The exons within the Dmloxl-2 gene that encode the copper-binding domain are split by the second intron in the same conserved position as all the human LOX genes, indicating an evolutionarily conserved genomic organization. The ORF of Dmloxl-1 encodes a protein of estimated molecular weight of 40.8 kD, and Dmloxl-2 encodes an estimated 57.8 kD product.

The DmLOXL-1 and DmLOXL-2 proteins have conserved C-terminal domains characteristic of all the lysyl oxidases, including the copper-binding site, residues of the lysine-tyrosyl quinone co-factor, and a cytokine receptor-like domain. DmLOXL-1 has one SRCR domain, and DmLOXL-2 has two SRCR domains. Predicted nuclear localization signals were identified within both DmLOXL-1 and DmLOXL-2, indicating that they might be involved in similar nuclear transport mechanisms as the human LOX (4, 7).

The human LOX and LOXL, which do not have SRCR domains, are known to be catalytically active amine oxidases. So far there is one report on the SRCR-containing LOXL4 that demonstrates that the presence of one, two, three, or all four SRCR domains, or the absence of all four domains, does not interfere with the amine oxidase activity of this protein (18). Based on these results, and the conserved C-terminal domains within DmLOXL-1 and DmLOXL-2, we anticipated that these too are active enzymes. Indeed, we have shown that the
secreted recombinant DmLOXL-1 is a BAPN-inhibited active amine oxidase.

Based on the signal sequence and secretion of recombinant DmLOXL-1 into media of Drosophila S2 cells transfected with expression plasmid pMT-Dmloxl-1, DmLOXL-1 might function as an extracellular amine oxidase. Does it fulfill functions analogous to the mammalian LOX proteins in the assembly of the extracellular matrix? There is no elastin in Drosophila, and there are no fibril-forming collagens with a long triple helix (53, 54). There are three conserved genes encoding two alpha chains of type IV collagen, and one gene encoding an orthologue of type XV/XVIII collagens (53, 54, 55). However, the substrates of DmLOXL-1 remain unknown.

Given the previous detection of the active LOX in the nuclei of cultured smooth muscle cells (4) that contributed to chromatin structural alterations (7); the presence of LOX and LOXL in the nuclei of several cell types (52); and the presence of the nuclear localization signal within DmLOXL-1, we performed immunohistochemistry using DmLOXL-1 antibody and localized DmLOXL-1 in the nuclei of Drosophila embryos and salivary gland cells in adult animals. In the salivary gland cells, DmLOXL-1 was also present in the cytoplasm and surface of these cells. The punctate staining pattern and the intense staining in the nuclei in embryonic tissues, that was reduced in adult tissues, was similar to the staining we noted for the nuclear LOX and LOXL proteins in mouse tissues (52).

Our previous data demonstrating down-regulation of several genes under DmLOXL-1 inhibitory conditions (38) and the current results further supported a nuclear function for DmLOXL-1. We noted localized, punctate DmLOXL-1 staining of the polytene chromosomes, however, without any obvious pattern. Though DmLOXL-1 is present in the nuclei, its association with the heterochromatin needs to be further studied as the staining we noted may be specific for polytene chromosomes.

The processed form of LOX was detected in the nuclei, but no previous studies reported nuclear localization for SRCR-containing mammalian LOX-like proteins. In these experiments, we could not determine if the nuclear DmLOXL-1 is full length, containing the SRCR domain, or a processed shorter form. There is a putative BMP-1 site within the human SRCR containing LOXL3, but only unpublished data supports some processing of this protein. Additional results demonstrated that the human LOXL4, lacking some, or all four SRCR domains, remains an active enzyme (18). Though in S2 cells we did not note processing of the recombinant DmLOXL-1, some form of processing remains a possibility in other cell types.

To further explore the nuclear role of Dmloxl-1 in vivo, we used the Drosophila PEV model. As DmLOXL-2 is expressed only in adults (38), we were able to address the function of DmLOXL-1 in early developmental stages. First, we searched existing Drosophila mutant strains for P-element insertional mutants. As no such strains were found for either of the Drosophila lysyl oxidase loci, we tested the in vivo effect of reduced DmLOXL-1 by raising the animals on BAPN supplemented media. BAPN inhibition of lysyl oxidases is never complete, low levels of enzyme activity have been shown to remain under in vitro inhibitory conditions (20, Fig. 4). Indeed, our activity measurements have shown similar kinetics. DmLOXL-1 retained a small amount (10%) of its activity under BAPN inhibition. In spite of this remaining activity, reduced DmLOXL-1 activity resulted in a significant developmental delay and a shift in sex-ratio. These results were in correlation with the earlier observed reduced transcription rates of multiple genes in BAPN-treated animals (38) and resembled the effect of increased heterochromatinization induced in mammalian cells by antisense LOX transfection (5).

PCR profiling revealed that the mRNA of Dmloxl-1 was present in the BAPN-treated larvae in all developmental stages. Therefore, the observed developmental delay may result from the inhibition of the activity of DmLOXL-1. It is not known how the Drosophila metabolize BAPN, and if the possible metabolites may contribute to the phenotype. However, specific in vivo BAPN inhibition of LOX was reported. LOX was specifically inhibited by BAPN concentrations as high as 400mg/kg body weight, injected intraperitoneally, twice daily, without
significant inhibition of monoamine oxidases (56).

PEV modification in Dmlox1-1 over-expressing strains and under inhibitory conditions indicated a nuclear role for the DmLOXL-1 protein. *Drosophila* male semilethality can result from increased sensitivity of males to chromatin changes. Females do not inactivate one of their X chromosomes, as mammals do, to achieve the same level of expression as for the autosomal genes (57). In contrast, males up-regulate the expression of genes from their single X chromosome (58). The Male Specific Lethal complex sequesters histone acetylase and chromatin binding proteins to the X chromosome in males, resulting in hyperexpression of the X-linked genes (59, 60, 61). We have also shown that in adult male animals Dmlox1-1 expression levels are significantly (*p* < 0.0001) higher than in females. Therefore, the development of males can be more sensitive to chromatin changes induced by DmLOXL-1 inhibition. Indeed, BAPN treatment caused less male flies to develop.

In *w*^*m4* Dmlox1-1/Dmlox1-1 transgenic animals, over-expression of DmLOXL-1 resulted in increase of the expression of the *w* gene, manifested as increased number of red eye cells, and increased amount of drosopertin indicating euchromatinization. Transgenic females produced higher amount of drosopertin than males, due to higher level of transcription. A large number of dominant PEV modifier mutations have been isolated in *Drosophila*, but most of these show effects only upon reduced expression levels (haplo-dependent), but no effects following up-regulation (triplo-dependent). A rare example for this haplo- and triplod-dependent behavior is provided by hel, an RNA helicase (62). DmLOXL-1 also demonstrates PEV modifying effect both upon reduced and increased expression, highlighting it as a valuable target gene for chromatin remodeling studies.

**ACKNOWLEDGMENT**

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**REFERENCES**

FOOTNOTES

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The abbreviations used are: LOX, lysyl oxidase; BAPN, \( \beta \)-aminopropionitrile; \( w^{md} \), white-mottled; PEV, position effect variegation; \( w \), white; SRCR, scavenger receptor cysteine-rich; rTE, recombinant tropoelastin.

Accession Numbers used are:
1 GenBank Accession Number M94054
2 GenBank Accession Number BT001394
3 GenBank Accession Number AI260913
4 GenBank Accession Number AC009349
5 GenBank Accession Number AC008287
6 GenBank Accession Number AC007803
7 GenBank Accession Number AC007802
8 GenBank Accession Number AJ295625
9 GenBank Accession Number AJ295626

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Table 1. Drosopterin concentration in transgenic Dmlox1-1/Dmlox1-1 males and females. Rows 1, 2, and 5 show controls, and rows 3-4 show double transgenic Dmlox1-1/Dmlox1-1 lines. Values show means of drosopterin pigment level with standard deviation.

<table>
<thead>
<tr>
<th>Genotype/strain</th>
<th>Drosopterin (mM)</th>
</tr>
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<tbody>
<tr>
<td>1 w</td>
<td>2.4 +/- 0.07</td>
</tr>
<tr>
<td>2 w\textsuperscript{md}; CyRoi/Sco</td>
<td>7.5 +/- 0.27</td>
</tr>
<tr>
<td>3 w\textsuperscript{md}/Y; Dmlox1-1/Dmlox1-1</td>
<td>12.3 +/- 0.43</td>
</tr>
<tr>
<td>4 w\textsuperscript{md}/w\textsuperscript{md}; Dmlox1-1/Dmlox1-1</td>
<td>25.4 +/- 0.98</td>
</tr>
<tr>
<td>5 Oregon-R</td>
<td>45.7 +/- 1.97</td>
</tr>
</tbody>
</table>

FIGURE LEGENDS

Figure 1. Chromosomal localization, transcriptional initiation sites, Northern blot analysis and gene structure of the Dmlox1-1 and Dmlox1-2 genes. Panel A: Chromosomal localization of the Dmlox1-1 and Dmlox1-2 genes. Upper figure, localization of Dmlox1-1 to 100C3-5; lower figure, localization of Dmlox1-2 to 58A. Dmlox1-1 corresponds to the gene symbol CG11335, and Dmlox1-2 to CG4402 in the Genome Annotation Database of Drosophila (GadFly). Panel B: Transcription start sites in Dmlox1-1 and Dmlox1-2. Left panel, a single start site of the Dmlox1-1 mRNA; right panel, two start sites for the Dmlox1-2 mRNAs detected by primer extension. Panel C: Northern blot analysis of Dmlox1-1 and Dmlox1-2 mRNA expression in adult Drosophila. rp49 cDNA was used as a positive control probe. Sizes of the mRNAs are indicated on the right side of the panel. Panel D: Genomic organization of Dmlox1-1 and Dmlox1-2 including size and relative position of exons and introns.

Figure 2. Amino acid sequence comparison of Drosophila DmLOXL-1 and DmLOXL-2 and human LOX and LOXL2. The N-terminal signal sequences are underlined, the SSCR, Cu\textsuperscript{2+}-binding, and cytokine receptor-like domains are boxed. Conserved residues are shaded and putative nuclear localization sequences are in bold. Double underlines in the Cu\textsuperscript{2+}-binding domain of Dmlox1-2 and the human lysyl oxidases (only Cu\textsuperscript{2+}-binding domains shown for LOX, LOXL3, and LOXL4) mark the amino acid that corresponds to the codon of the conserved splice site. Splicing occurs after the second nucleotide of the respective codon. Vertical line in the lysine tyrosylquinone co-factor linkage domain shows the conserved splice site in the Drosophila lysyl oxidases.

Figure 3. Localization of DmLOXL-1 in embryos and adult salivary gland cells. Immunostaining of 0-6 hrs embryos, using lamin Dm0 (A) and both lamin Dm0 and DmLOXL-1 (B) antibodies. Lamin Dm0 antibody staining of the nuclear lamina; intense, punctate DmLOXL-1 staining within the nuclei of these cells. In adult salivary gland squashes RNA polymerase II antibody strongly stained the polytene chromosomes (C), shown next to transparent light control (D). Punctate DmLOXL-1 staining on these chromosomes (E, short arrows). Nuclear, cytoplasmic and pericellular DmLOXL-1 staining in adult salivary gland cells (E, long arrows). Preimmune serum control (F). The Lamin Dm0 ADL67 antibody dilution was 1:1000, the pre-adsorbed DmLOXL-1 antibody was 1:300. Secondary antibodies were Alexa Fluor 546 goat anti-mouse IgG and Alexa Fluor 488 goat anti-rabbit IgG. Embryos were mounted on slides, with Vectashield mounting medium for...
fluorescence with DAPI and visualized on Zeiss LSM5 Pascal confocal microscope using x100 oil immersion objective. Polytenic chromosome squashes were from third-instar larvae salivary glands. In the experiments DmLOXL-1 antibody and pre-immune serum were used in 1:250 dilutions. RNA II polymerase antibody, was diluted 1:500. Fluoresceine i-thiocyanate-conjugated anti-rabbit secondary antibody Alexa 488 was diluted at 1:300. Fluorescence of the tissue specimens was photographed using FITC filter.

**Figure 4. BAPN inhibition of recombinant DmLOXL-1.** BAPN inhibition of recombinant tropoelastin (rTE) oxidation by recombinant DmLOXL-1. Arrowhead shows the BAPN concentration (371nM) required for 50% inhibition of activity of 67.5nM DmLOXL-1.

**Figure 5. Altered sex ratio in BAPN treated Drosophila.** Male to female ratios of *Drosophila* reared on normal food (control) and food containing 5 mM BAPN. The error bars show the standard deviations of each sample group.

**Figure 6. Real time RT-PCR analysis of the expression level of Dmloxl-1 in normal and transgenic animals.** A: Basal transcription levels in transgenic *Dmloxl-1/Dmloxl-1* males and females. B: Transcription levels in non-transgenic heat-shocked controls. C: Elevated transcription levels in transgenic heat shocked *Dmloxl-1/Dmloxl-1* animals. Expression levels were normalized for *rp49*. Statistical analysis was done using Unpaired t-test. Error bars represent standard deviation.
Figure 1. Molnar et. al.
Figure 3. Molnar et al.
Figure 4. Molnar et. al.
Figure 5. Molnar et al.

% of total flies

- **Females**
  - Control: 48.8%
  - BAPN treated: 61.8%

- **Males**
  - Control: 51.2%
  - BAPN treated: 38.2%
Figure 6. Molnar et. al.

- Male
- Female

Relative level of expression

A
B
C

p < 0.025
Drosophila lysyl oxidases Dmloxl-1 and Dmloxl-2 are differentially expressed and the active DmLOXL-1 influences gene expression and development
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