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Beadex, a *Drosophila* LIM domain only protein, function in follicle cells is essential for egg development and fertility

Subhash Kairamkonda, Upendra Nongthomba*

Department of Molecular Reproduction, Development and Genetics, Indian Institute of Science, Bangalore 560012, India

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ABSTRACT

LIM domain, constituted by two tandem C2H2 zinc finger motif, proteins regulate several biological processes. They are usually found associated with various functional domains like Homeodomain, kinase domain and other protein binding domains. LIM proteins that are devoid of other domains are called LIM only proteins (LMO). LMO proteins were first identified in humans and are implicated in development and oncogenesis. They regulate various cell specifications by regulating the activity of respective transcriptional complexes. The *Drosophila* LMO protein (dLMO), Beadex (Bx), regulates various developmental processes like wing margin development and bristle development. It also regulates *Drosophila* behavior in response to cocaine and ethanol. We have previously generated *Bx* null flies and shown its essential function in neurons for multiple aspects of female reproduction. However, it was not known whether *Bx* affects reproduction through its independent function in ovaries. In this paper we show that female flies null for *Bx* lay eggs with multiple defects. Further, through knock down studies we demonstrate that function of *Bx* in follicle cells is required for normal egg development. We also show that function of *Bx* is particularly required in border cells for *Drosophila* fertility.

1. Introduction

Protein-protein interactions are essential for several biological processes and are facilitated by specialized motifs. LIM domain is one such structure which facilitates protein-protein interactions [1]. LIM domain, named after its discovery in three members of this group (Lin-11, Isl1 and Mec3), is constituted by tandem organization of two zinc finger motifs containing conserved cysteine-histidine residues that coordinate zinc binding [2]. LIM domain was discovered initially associated with 3 homeodomain containing proteins [3–5]. Later it was discovered to be associated with several homeodomian proteins in various organisms which are together grouped as LIM-homeodomain (LIM-HD) proteins. This class of proteins play important role in developmental processes like cell and organ specification: *Lin-11* and *Mec3* in *Caenorhabditis elegans* are essential for vulva and neuronal development respectively [4,6], *Isl1* in mice is essential for various cell specifications [7,8], Apterous (Ap) in *Drosophila* is important for the wing margin development [9] and LHX-6 and LHX-8 in mouse are involved in the development of various tissues [10–12]. Studies in *Xenopus* demonstrated that the LIM domains in these proteins help in the formation of functional complexes with other interacting proteins, without which the normal function of LIM-HD proteins is abrogated [13,14]. By regulating activity of the LIM-HD proteins, LIM domain affects target gene expression. However, LIM domains also play role in other cellular processes like signal transduction and cytoskeleton remodeling by associating with various functional domains like kinase domain (LIM kinases) [15,16] and protein interacting domains like SH3 domain (LIM and SH3 domain protein 1 (LASP1)) [17] and actin binding domain (Actin binding LIM (ABLIM) protein, [18]) (reviewed in [19]).

LIM domains also occur independently in proteins without the combination of any other functional domains. These proteins are termed as LIM domain only (LMO) proteins. Four LMO proteins (LMO1–4) are identified in humans (reviewed in [20]). LMO1 and LMO2 were identified associated with T-cell acute lymphoblastic leukemia (T-ALL), wherein their expression was enhanced [21,22]. They were established as oncogenes following induction of T-cell leuckemia through their over-expression in transgenic mouse [23]. LMO3 and LMO4 were also implicated in different cancer conditions [24,25]. However, LMO proteins play important role in development as well: LMO2 is essential for haematopoiesis and angiogenesis, while LMO4 is involved in interneuron development [26–28]. Since LMO proteins do not contain any other domains, they execute their function by acting as scaffold and facilitator of multi-protein interactions. For example, LMO4 helps in the formation of active LMO4-SCL-Gata2-NIL transcription protein complex that induces target gene expression to promote specific interneuron fate in mice [28].

* Corresponding author.
E-mail address: upendra@iisc.ac.in (U. Nongthomba).

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Following discovery of the vertebrate *LMO* genes, three studies independently identified *Drosophila* homologue of *LMO*, termed *dLMO* or *Beadex* (*Bx*) [29–31]. This greatly helped in understanding not only the cellular and biological processes affected by LMO proteins in general, but also the mechanistic insights of their mode of function. *Bx* gain of function mutant shows beaded wing margins [32]. Apterous (Ap), a LIM-HD protein interacts with Chip to form a functional protein complex that is essential for formation of normal wing margin. However, in *Bx* hypermorphs (like Bx^{1} or Bx^{J}), due to enhanced availability of Bx, Bx binds to LIM binding doman (LBD) of Chip and reduces the activity of Ap-Chip complex [31], leading to development of defective wing margin. Bx also binds to another transcription factor Pnr and positively regulates transcription of *Achaete* and *Scute* involved in dorsal thoracic bristle development [33,34]. Apart from development, *Bx* also affects behavior response of *Drosophila* exposed to ethanol or cocaine – *Bx* gain of function mutants are more tolerant to the affects of cocaine and ethanol [35–37]. This function of *Bx* in neurons is evolutionarily conserved, since mice with altered expression of *LMO3* or *LMO4*, the vertebrate homologues of *Bx*, also show similar behavior response upon treatment with ethanol or cocaine [37,38]. *Bx* is also shown to regulate the feeding behavior by controlling the passage of food in digestive tract [39]. Active role of *Bx* in such diverse processes further emphasizes the possibility of its involvement in many other biological processes. With the isolation and characterization of null allele of the *Bx*, we have demonstrated previously that *Bx* function in neurons is essential for several aspects of female reproduction like ovulation, fecundity, fertility, sperm release and egg deposition [40]. However, it was not clear if the reproductive defects observed in *Bx* null females were exclusively through its function in neurons or attribution from additional function in ovaries is also required. In this paper, we demonstrate that *Bx* expression in *Drosophila* ovary, especially follicle cells and border cells, is essential for fecundity, fertility and normal egg development.

2. Materials and methods

2.1. Fly stocks

All the flies were maintained at 23 ± 2 °C, under 12 h Light/Dark (L/D) cycles on cornmeal-sucrose-yeast agar media. Canton-S (CS) and w^{1118} flies were used as wild type controls, where ever not specified. Bx^{7}, a *Bx* null allele generated through p-element mobilization was described earlier [40]. *Bx*-RNAi lines and *Bx*-TRiP lines were obtained from Vienna *Drosophila* RNAi Centre (VDRC Transformant ID# 2917) and Bloomington *Drosophila* Stock Centre (BDSC, BS#29454, BS#35637, BS#57465) respectively. Following Gal4 fly stocks were obtained from Bloomington *Drosophila* stock centre, Indiana- neurons specific Gal4 (Elav-Gal4 with UAS-Dcr2, BS# 25750) germline specific Gal4 (*nanos*-Gal4 with UAS-dicer2 (BS# 25751, [41], follicle cells Gal4 (c323a (BS#3732), c204 (BS#3751), c306 (BS#3743), [42] and border cells specific Gal4 (*slbo*-GFP (BS# 6458), [43]. Sensory organ precursor Gal4, pnr-Gal4 [33] and UAS-GFP were procured from National Center for Biological Sciences (NCBS) stock centre, Bangalore, India.

2.2. Fecundity and fertility assays

Three days old virgin females of Gal4 lines or promoter enhancer trap lines were crossed with virgin males of *Bx*-RNAi line and kept at 29 °C. Female progenies from these crosses were aged for 3 days and allowed to mate with 2–3 days old virgin Canton-S males for 12 h. Post mating, females were transferred to egg laying media, consisting of 1% sucrose supplemented with yeast paste, and assessed for fecundity and fertility (as described in [40]).

2.3. Imaging studies

In order to check the expression profile of Gal4 and enhancer trap lines in ovaries, females of these lines were mated with UAS-GFP males and grown at 29 °C. Ovaries of 3–4 days old mated female progeny from these crosses were dissected in PBS and processed for confocal imaging (as described in [40]). Images were acquired on Zeiss LSM510 meta confocal microscope and processed with LSM software (version 3.2.0.115, Zeiss). For assessing border cells migration ovaries from mated females of control, *Bx* knock down and Bx^{7} mutant lines, all expressing *slbo*-GFP, were dissected in PBS and processed for confocal imaging. Stage 10 egg chambers were imaged and examined for border cells migration. For assessing egg morphology, fresh eggs (within 2–3 h) laid by control and test females were isolated and imaged on Olympus SZX-12 stereo microscope using Leica Application Suite (LAS, v3.8).

2.4. Molecular work

To check expression of *Bx*, RNA was isolated from ovaries of 3–4 days old females of wild type (CS) and *Bx* null, Bx^{7}, using Trizol (Sigma Aldrich, Bangalore) by following manufacturer's instruction. cDNA was prepared from 2 µg of isolated RNA using first strand cDNA kit following manufacturer's protocol (Thermo Fischer Scientific). *Bx* was amplified using following primers *Bx*FP 5' – CAGCCTTCGAGATGGTG ATG – 3' and *Bx*RP 5' – AAGGAGGAGACGTGTCGCTT – 3'. *rp49*, a ribosomal protein coding gene, was used as control using following primers *rp49*FP 5' - TTCTACCAGCTTCAAGATGAC – 3' and *rp49*RP 5' - GTGTATTCCGACCACGTTACA – 3'.

2.5. Data plotting and statistical analysis

GraphPad prism 5 was used for plotting data, graph preparation and statistical test analysis.

3. Results

3.1. Beadex null females show defective egg structures

In order to assess the role of Bx in ovaries, we first checked for its expression. RT-PCR analysis showed that *Bx* was expressed in wild type ovaries, while the expression was lost in ovaries of Bx^{7}, a *Bx* null mutant (Fig. 1A). Bx^{7} females show normal stages of ovariole development [40]. However, some of the eggs laid by *Bx* null females show few easily

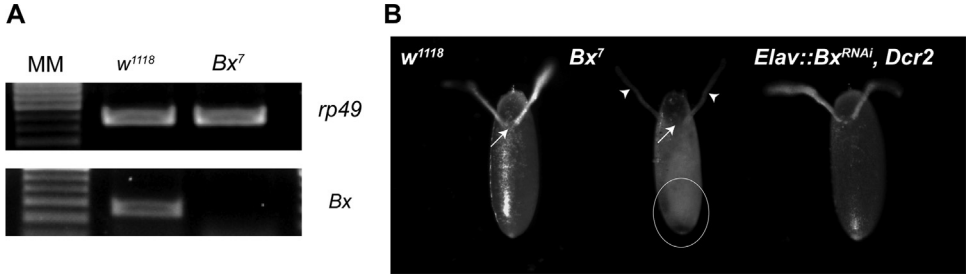

Fig. 1. *Bx* is expressed in ovaries and affects egg morphology. (A) Reverse transcriptase PCR analysis revealed that *Bx* was expressed in ovaries and this expression was lost in Bx^{7} mutant ovaries. (B) Eggs laid by Bx^{7} mutants showed abnormal morphology where in the dorsal appendages (arrow heads) were thin and wavy. Also the distance between the bases of two dorsal appendages on egg (arrow) was wider on eggs laid by Bx^{7} mutant compared to that of wild type. Eggs laid by mutants were translucent (circled region) compared to opaque nature of controls. These defects were not observed upon knocking down of *Bx* in neurons using VDRC *Bx*-RNAi.