Beadex, the LIM Only protein in *Drosophila* is required for Indirect Flight Muscle function.

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Abstract- LIM domain proteins are involved in diverse biological processes, such as, development and differentiation, by assisting in protein- protein interaction. *Beadex*, the LIM only protein in *Drosophila*, is coded by *dLMO* gene. Beadex has two tandemly repeated LIM domains. *Beadex* is normally expressed in the wing pouch of the third instar wing imaginal disc during patterning. Initial studies with *Bx* gain of function mutants showed that over-expressed Beadex can bind to Chip and thereby interfere with formation of a functional complex between Apterous and Chip during wing development. *Beadex* localizes in nucleus in early stages of muscle development. Present study is to understand the role of *Beadex* in muscle function. We show that Beadex is necessary for survival and proper functioning of the Indirect Flight Muscle in *Drosophila*.

Index Terms- LIM only protein, Beadex, Indirect Flight Muscles.

I. INTRODUCTION

LIM domains are evolutionarily conserved protein motifs in eukaryotes implicated in wide variety of biological functions. The acronym LIM is taken from the initials of the proteins where it was first identified (Lin-11from *Caenorhabiditiselegans*, Isl-1 from rat, and Mec-3 from *Caenorhabditis elegans*) (Way *et al*., 1988; Karlsson *et al*., 1990). LIM domain comprises of a cysteine rich consensus sequence, which adopts a topology wherein its eight highly conserved residues coordinate two zinc ions. They function as modular protein binding interface allowing protein–protein interactions to occur (Schmeichel and Beckerle, 1994). Diverse biological roles played by LIM proteins include cellular differentiation (Akazawa *et al*., 2004), cell specification (Thaler *et al*., 2002), actin dynamics (Maul *et al*., 2003), neuronal path finding (Terman *et al*., 2002), and cellular adhesion and signaling (Kasai *et al*., 2003). These diverse roles played by LIM proteins are attributed to the fact that they are found linked to wide variety of other protein domains such as homeodomains, catalytic domain, cytoskeletal binding domains and many other protein motifs (Kadrmas and Beckerle, 2004).

Beadex (Bx), the LIM only gene in *Drosophila* is known to play important role in wing development by regulating *Apterous (Ap)* activity levels (Milan *et al*., 1998; Shoresh *et al*., 1998). Biochemical experiments have shown that both Bx and Ap compete for Chip, another LIM domain protein. Binding of Bx to Chip interferes with formation of a functional complex between Ap and Chip, thus modulating Ap activity levels It is also known to express in the primary circadian pacemaker neurons in brain wherein it regulates the behavioural responses to cocaine (Tsai *et al*., 2004). Recent studies have also shown that *Bx* is a positive regulator of transcription during thoracic bristle formation (Zenvirt *et al*., 2008). Bx plays an important role in female reproduction through the motor neurons (Kairamkonda *et al*, 2014).

Many groups have isolated *heldup-a* (*hdp-a*) mutations which were hypomorphic mutants in *Bx* locus showing abnormal wing position (Fahmy and Fahmy, 1958; Lifschytz and Green, 1979) and flightlessness (Shoresh *et al*., 1998). These mutants could suppress hypermorphic phenotype of the *Beadex* allele. Since none of these studies looked at the muscle function, we took up this opportunity to study the role of *Bx* in the Indirect Flight Muscles (IFM). *Drosophila* IFM is a well-studied and understood model system to study muscle development. (DeSimone *et al*., 1996, Nongthomba and Ramachandra, 1999). Studies from our lab have also suggested that the flies lacking *dLMO* are flightless revealing that *Bx* may play an important role in the IFM development and function. In the present study, we have utilized genetic and cellular approaches to study the *Bx* gene function. However, its interacting partners in IFM and pathway involved remains to be elucidated.

II. MATERIALS AND METHODS

Fly strains:

All the flies were maintained at 25°C in Cornmeal-yeast-agar media. *Canton-S* and w^{1118} were used as control flies unless mentioned. *UAS-dLMO* was a gift from Stephen Cohen (Singapore). *Mef2-Gal4* was provided by Prof. John Sparrow (York University, UK). Bx^{7} allele was generated and characterized in the lab (Kairamkonda *et al*, 2014).

Flight test:

Flight test was performed as described by Drummond *et al*., 1991. Briefly, flies were released into Sparrow box, a plexiglass container with light source at the top, to determine their flight ability to fly up, horizontal, down, or the inability to fly, Flightless.

Polarized light microscopy:

Fly hemithoraces were prepared for polarized microscopy as described in Nongthomba and Ramchandra, 1999. Briefly, fly thoraces were frozen in liquid nitrogen, bisected longitudinally

using a razor blade, dehydrated in alcohol series, cleared in methyl salicylate and mounted using DPX mounting medium. The hemithoraces were observed in Olympus SZX12 microscope and photographed using Olympus C-5060 camera under polarized light optics.

Immunocytochemistry:

Desired tissues were dissected, fixed in 4% paraformaldehyde and washed with phosphate buffered saline containing 0.3% Triton-X (PBTx). Blocking was done in 2% horse serum for 1hour. Samples were then incubated in primary antibody overnight at 4°C rocking. Post incubation in primary antibody, samples were washed thrice with PBTx for 10min each followed by blocking in horse serum for 1hr. Incubation in secondary antibody was done for 2hrs at room temperature. Secondary antibody tagged with appropriate fluorochrome (Molecular probes) was used in 1:1500 dilution. Washing with phosphate buffered saline containing 0.05% Tween-20 was done three times for 10min each. Finally, tissue samples were mounted in mounting media vectashield (Vector laboratories H-1000). Rat Anti–dLMO antibody was given by Stephen Cohen and was used in 1:50 dilution (Milan *et al*., 1998). Anti-Twist antibody for myoblast staining was diluted to 1:2500 (Gift from Martin Milner, St. Andrews University, UK). Anti-Erect wing antibody (1:500) (Sudipto and Vijayaraghavan, 1998) was used to track the myoblast nuclei in pupal stage. *Phalloidin-TRITC* (Sigma Aldrich) staining was done to counterstain the F-actin in muscles. Confocal images were acquired using a laser scanning confocal microscope (LSM 510, Carl Zeiss).

Transmission Electron Microscopy:

Adult hemi-thoraces were dissected in phosphate buffered saline (PBS, pH 7.2) and fixed overnight at 4°C in 3% glutaraldehyde (Electron Microscopy Sciences) in sodium cacodylate buffer, pH 7.14. Tissues were washed with sodium cacodylate buffer (x2, 15 minutes), and then fixed with 1% osmium tetroxide in buffer (90 minutes), dehydrated through alcohol series, en-bloc stained with 2% uranyl acetate in 95% ethanol (1 hour), followed by final dehydration in absolute ethanol (x2, 30 minutes). Clearing was done in propylene oxide (x2, 15 minutes) to facilitate infiltration. Infiltration was done by keeping the tissues in 1:1 propylene oxide: Epoxy Resin (overnight), followed by three changes (3x3 hours) in embedding medium. Tissues were oriented and kept at 60°C for 48 hours for resin polymerisation. Sections were cut (Leica EM UC6), stained with uranyl acetate for 2 hours, washed in distilled water, dried and stained with lead citrate for 5-7 minutes and finally washed. Images were captured using an FEI Tecnai electron microscope.

Reverse transcriptase PCR (RT-PCR):

Two days old flies were collected and frozen in 70% alcohol in -80°C. IFM were dissected from these flies and total RNA was isolated using Sigma Trizol reagent using manufacturer's protocol. Complementary DNA (cDNA) was prepared using Fermentas first strand cDNA kit. Primers used for the study are enlisted in Table 1. Fermentas 2X PCR mix was used for the PCR amplification. RT-PCR primers used are rp49F - TTCTACCAGCTTCAAGATGAC, rp49R – GTGTATTCCGACCACGTTACA, Bx-RA-F-CTAATTGAGTCGAGTGTGCGTG, Bx-RA-R-ctgctgctgttggttggaggaa,Bx-RB-F-GGAGTACCTCTACAACGCTA, Bx-RB-R-GGTACCCATGCCCAGGGCTTTATT.

III. RESULTS AND DISCUSSIONS

Expression profiling of *dLMO* in developing IFM.

To understand the gene function we need to know whether the gene is expressed in the tissue of interest. Both the major isoforms *Bx-RA* and *Bx-RB* were present in Dorsal Longitudinal Muscles, a subset of IFM, when *Bx* isoform specific primers were used. (Figure 1 A). Then to know whether Bx is present during IFM development, we resorted to antibody staining specific to dLMO protein. Previous reports have shown *Bx* expression in wing imaginal disc where it regulates the dorsal–ventral (DV) patterning of the wing. However, its role in muscle development was not studied so far even though *heldup-a* (*hdp-a*) mutations isolated in *Bx* locus showed flightlessness (Shoresh *et al*., 1998).RNA in situ hybridization for *Bx* in wing imaginal disc showed *Bx* expression in the wing disc notum (Asmar *et al*., 2008). Myoblasts which give rise to IFM are also present in the notum region of the wing imaginal disc (Bate *et al*., 1991; Fernandes and VijayRaghavan, 1993; Roy and VijayRaghavan, 1998). Antibody staining for dLMO in wing imaginal disc revealed that it is expressed in the myoblasts and colocalizes with twist (*twi*), a bHLH transcription factor expressed in all the adult myoblasts (Figure 1 B-B").

Myoblasts in the wing imaginal disc fuse to the larval oblique muscle (LOM) during pupal metamorphosis to give rise to DLM (Fernandes *et al*., 1991). Since myoblasts adhered to wing imaginal disc stained positive for Bx in IHC, we followed the expression in the IFM to check if Bx expresses in developing myofibre. Since vertebrate LMO1-4 genes show nuclear expression (Kadrmas and Beckerle, 2004; Joshi *et al*., 2009) we wanted to check if Bx also shows nuclear expression. Colocalization of Bx was done with Erectwing (EWG), which is a known nuclear transcription factor. EWG is a good marker for nuclear staining in developing IFM and expresses during a very short window of IFM development (Roy *et al*., 1998). Bx predominantly localizes in the cytosol and does not colocalize with EWG in developing IFM. However, nuclear expression of Bx could be seen in the unfused myoblasts at 24hrs APF (Figure 1 C-C''). The horizontal section (Figure 1 D-D") of the IFM shows parallel arrangement of the myofibrils. Bx does not show any colocalization with the *Phalloidin-TRITC* stained myofibrils nor does it appear to be localized to the sarcomeric structure. The staining could also be seen prominently in the space which demarcates the two bundles of myofibres such kind of cytosolic localization of the LIM protein in muscles has not been reported in *Drosophila*.

***Beadex* null allele shows muscle defects.**

Bx hypomorphic alleles were generated as described in Kairamkonda *et al*., 2014. Out of many lines generated Bx^7 was null for both the isoforms *Bx-RA* and *Bx-RB* (Kairamkonda *et al*., 2014) and showed the wings up phenotype. When the fight test was performed the Bx^7 flies were completely flightless when

compared to wildtype counterparts. So this drove us to look for any defect in the IFMs of the null flies. The IFMs of Bx^7showed six normal muscle fascicles under polarized light microscopy (Figure 2 A-A'). The confocal images (Figure B-B') showed normal sarcomere structures when stained for actin. Electron micrographs (Figure 2 C' and C'') show muscle defects at the ultrastuctural level. The white arrows indicate that there are missing myofilaments in the sarcomere and the black arrow indicate that there is distorted M-line in Bx^7 compared to normal arrangement in wildtype flies (Figure 2C). This suggests that *Bx* is important in structural maintenance and function. It was also noticed that the mutant flies have less longevity than wildtype flies. Further studies can reveal how this protein interacts with other proteins to restore muscle structure and function.

Targeted overexpression of the *Bx* in IFM leads to muscle deformities.

Mef2-Gal4 expresses very strongly in the adult muscles including IFM (Roper *et al*, 2005). Overexpression of Bx using single copy of *Mef2-Gal4* showed very severe phenotype and none of the individuals could survive until adulthood. Pupae inside the pupal case showed abnormal development at early stage and later shrink in size and do not develop further. Late stage pupae were dissected to expose their IFM. Sarcomeric organization of the DLM fibre stained with Phalloidin–TRITC shows defects in Z-disc (Figure 3 B-B'') compared to the wildtype flies (Figure 3A). Many muscle mutants show defects in the sarcomeric organization. However, reasons for muscle deformities arising because of altering the levels of a cytosolic localized protein Bx requires elaborate study on the role of this protein in the development of Indirect Flight Muscles in *Drosophila.*

IV. CONCLUSION

Bx gene consists of two LIM domains which are highly conserved in the higher vertebrates including humans. Overexpression of the LMO genes in humans cause T cell acute leukemia (Boehm *et al*., 1991; Rabbitts *et al*., 1998). Similar results have been demonstrated in transgenic mice and zebra fish overexpressing LMO transgene (Larson *et al*., 1994; Langenau *et al*., 2005). Our result show that Bx expression starts in the myoblasts and continues in the developing and adult IFM. However, unlike these known LIM proteins in *Drosophila*, *Bx* is not adhered to muscle fibre and is localized in cytosol.

Previous studies on wing morphogenesis reveal that *Bx* regulates the activity level of *Ap* by competing with *Chip*. However, *Bx* may not be interacting with same partners during IFM development as *Ap* misexpression in the IFM inhibits muscle differentiation. *Ap* expression is also deregulated in IFM in Vestigial (*Vg*) null mutants leading to muscle apoptosis (Bernard *et al*., 2003). Since altering *Bx* levels lead to sarcomeric defect we believe that unlike *Vg* mutants, *Ap* is not deregulated in *Bx* mutants, otherwise it would have shown muscle differentiation defect leading to degeneration as seen in IFM overexpressing Ap. Nevertheless, *Ap* is required for proper development of the IFM through epidermal cues and functions as the selector gene for the formation of direct flight muscles (DFMs) (Ghazi *et al*., 2000). *Bx* certainly is not involved in similar pathway as structural proteins form sarcomeres albeit with some defect. However, we need to check *Ap* expression in *Bx* mutant background.

Mechanical overload in muscles lead to activation of a host of downstream signaling pathways (Chien 1999; Pan *et al*., 1999; Seko *et al*., 1999). Mechanical stretch sensors that sense these biomechanical strains and transduce downstream effectors are largely unknown. Identification of the components of stretch activation is of great clinical importance, because these mechanisms are part of the adaptive response to cardiac disease and heart failure. Work done on MLP knockout mice has revealed that they are a key component of the muscle stretch sensing machinery (Knoll *et al*., 2002). MLP associates with T-cap and are structural component of the sarcomere and MLP deficient mice develop dilated cardiomyopathy with hypertrophyand heart failure after birth. Similar impaired mechano-sensory stress signaling was found in case of missense mutation in *CSPR3* which codes for MLP and segregates with HCM (Geier *et al*., 2008). Since altering cytosolic localized LIM protein *Bx* levels in *Drosophila* IFM lead to sarcomeric defect, hence one can speculate role of *Bx* in similar kind of mechanical stretch sensing. There is similar report from zebrafish mutant *main squeeze* (*msq*), which supports that downregulation of the stretch-responsive genes leads to muscle defect and heart failure. *msq* is the mutation in *integrin-linked kinase*(*ilk*) gene which specifically localizes to costameres and sarcomeric Z-discs (Bendig *et al*., 2006).

Present study reports that *Bx* has two isoforms present in the IFMs. In early stages of muscle development *Bx* shows nuclear localization in myoblasts and later in development it is cytosolic in nature. Mutant flies null for *Beadex* show muscle deformities suggesting an important role in IFM maintenance. Over-expressing Bx shows Z disc deformities and pupal lethality confirming its role in muscle development. Further controlled temperature shift and protein interaction experiments can still reveal the larger role played by *Bx* in *Drosophila* muscle development.

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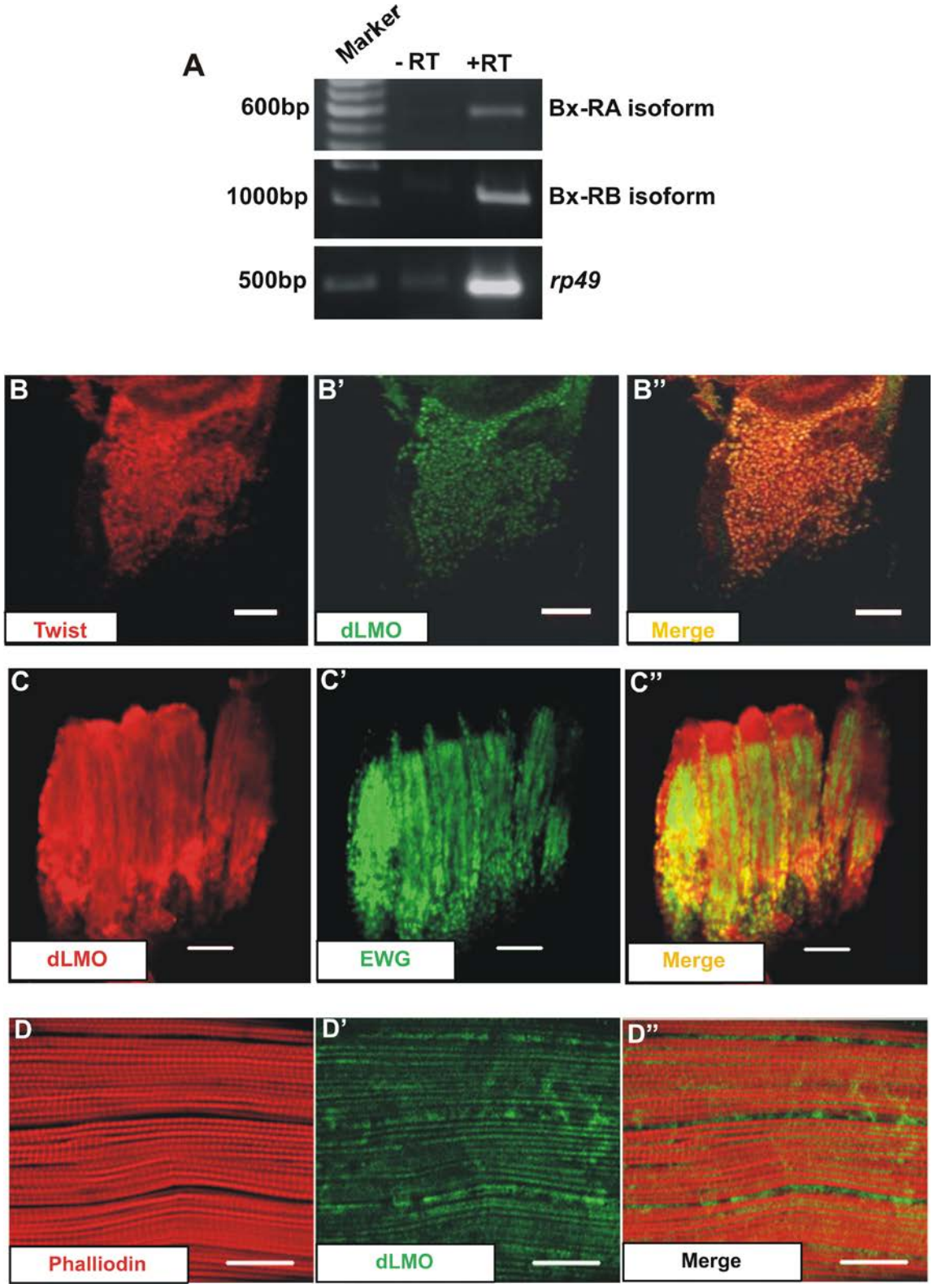

Figure 1. Expression profile of *dLMO* in developing IFM. (A) RT-PCR from IFM showing expression of both the isoforms of *Bx*. *rp-49* serves as the control. (B-B'') Double antibody staining with Twi and Bx (dLMO) in the wing disc notum showed that both the proteins colocalize in the wing disc myoblasts. Boundary denotes the myoblasts in the notum region (Scale bar~30µm). (C-C'') EWG is transcription factor which specifically localizes in the nucleus. Arrows indicate that Bx localization is less pronounced in nucleus than cytosol of developing IFM. Pupal preparations are of 24hrs APF. (Scale bar~30µm). (D-D") Horizontal section of IFM showing myofibrils arranged in parallel bundles stained with *Phalloidin-TRITC*. *Bx* is not localized in myofibrils but is dispersed along the space between myofibrils and fibres (arrows) (Scale bar 30µm).

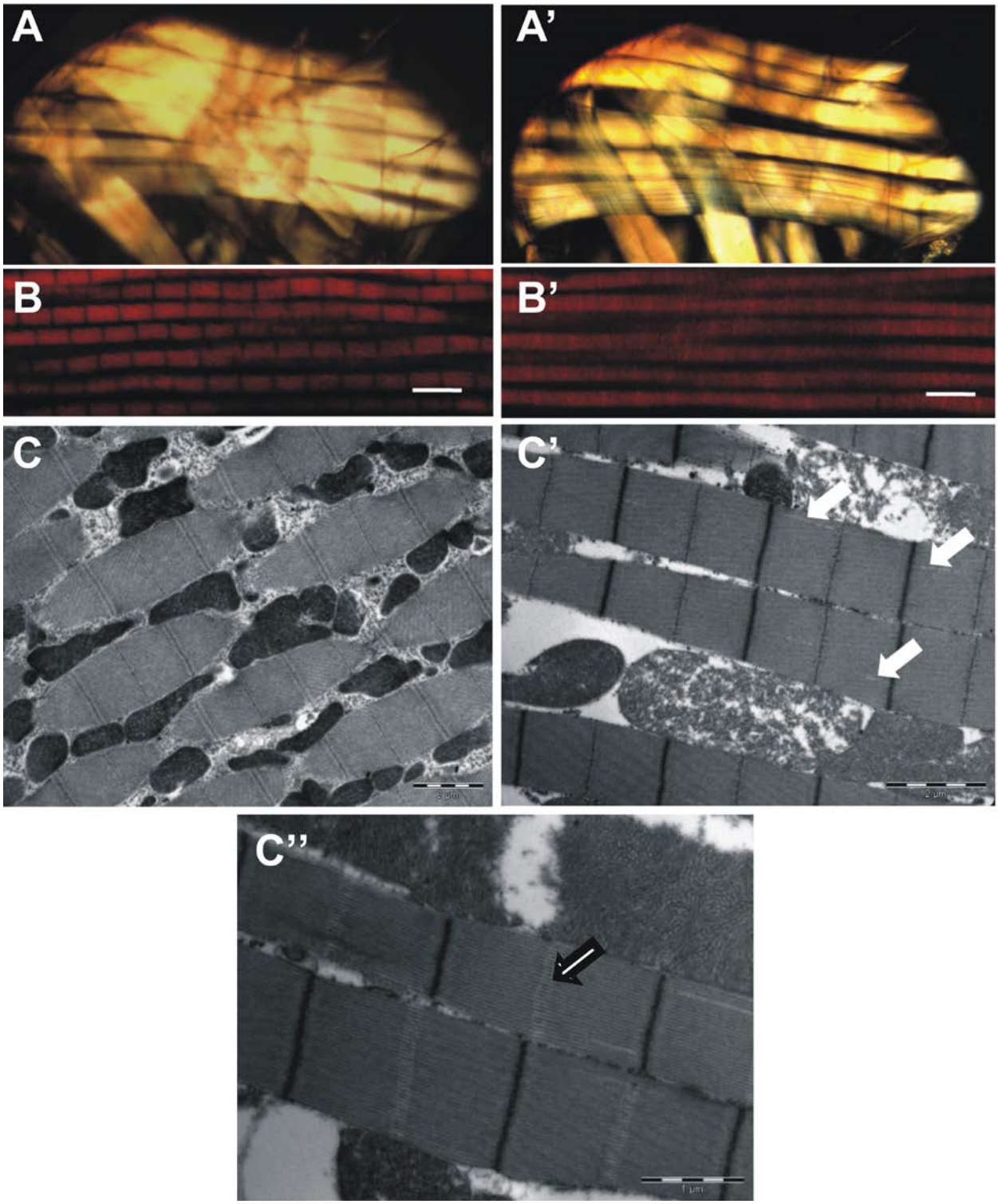

Figure 2: ***Beadex* null mutant shows muscle deformities.**
(A-A') Polarized images showing normal IFM of w^{1118} (A) and Bx^{7} (A'). (B-B') Confocal images show normal myofibrillar arrangement in both w^{1118} (B) and Bx^{7} (B'). Muscles are stained with *Phalloidin-TRITC*. (Scale bar~5µm). (C-C'') Electron micrographs show (C') abnormal sarcomeres (white arrows) and (C'') shows disrupted M-line (black arrow) compared to wildtype (C).

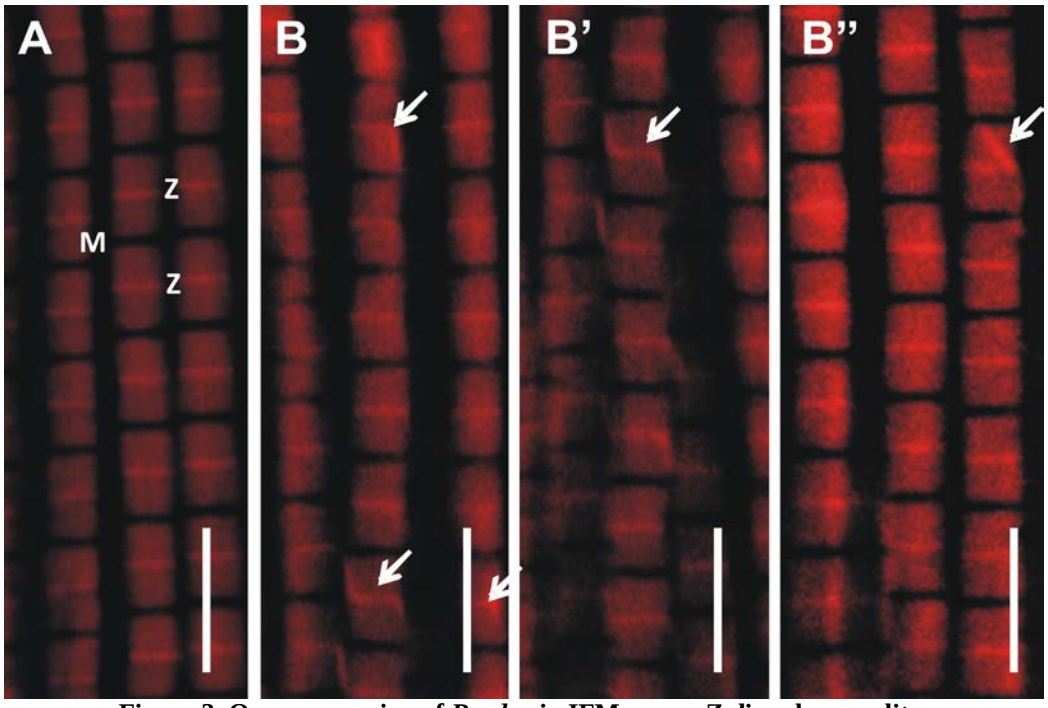

Figure 3: **Over-expression of *Beadex* in IFM causes Z disc abnormality.**
(A-B'') Over-expression of *Bx* using *Mef2-Gal4* leads to deformities in IFM. B-B'' shows spikes and abnormal Z-disc (white arrows) when *Bx* is over-expressed compared to Control (A). Muscles are stained with *Phalloidin-TRITC.* Arrows point to abnormal Z-discs (Scale bar~5µm).