Biochemical and Biophysical Research Communications 438 (2013) 329–333

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

The embryonic transcription factor Zelda of *Drosophila melanogaster* is also expressed in larvae and may regulate developmentally important genes

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ARTICLE INFO

Article history:
Received 7 July 2013
Available online 24 July 2013

Keywords:
Drosophila melanogaster
Zelda (Zld)
Larval wing disc

ABSTRACT

The transcription factor Zelda plays a pivotal role in promoting the maternal to zygotic transition during embryogenesis in *Drosophila melanogaster*. However, little is known about its role later in development. Here we are showing that Zelda is essential for proper wing development through gain and loss of function experiments. *Zelda's* transcript variants RB, RC and RD are present in imaginal wing discs of third instar larvae and the production of 2 protein isoforms of ~180 and ~70 kD was detected in the same tissue. In ChIP experiments using larval wing discs, Zelda was found to bind to a region of the *optomotor-blind* gene, suggesting an interaction with a Dpp target that promotes wing growth and patterning.

1. Introduction

The *Drosophila* zinc finger transcription factor Zelda (Zygotic early *Drosophila* activator, Zld) appears to play a major role as a regulator of genome activation in the earliest stages of *Drosophila* development. It was shown to bind to a sequence motif referred to as a TAGteam site and it was demonstrated to function as a key transcriptional activator during the maternal-to-zygotic transition – MZT [1]. ChIP-Seq data revealed Zld's binding to a significant number of these sites during embryonic development and marks regions that are later bound by zygotically expressed transcription factors, suggesting that it can also be part of the machinery that controls the sequence of events following the MZT [2,3]. Moreover, it has also been proposed that Zelda ensures coordinated gene expression during embryonic development either by increasing chromatin accessibility for several other transcription factors, or by being involved in the polymerase pausing mechanism [3]. However, its exact mechanism of action during embryogenesis remains to be further elucidated.

Zelda was also shown to be expressed in larval and pupal stages [4] indicating a possible involvement in the regulation of molecular events throughout the developmental process. However, no data exist to date regarding its potential function in post embryonic development.

In this study, we found that overexpression or knock down of *zld* in larval wing discs using the GAL4/UAS system, resulted in strong impaired phenotypes, an indication of its essential role in wing growth. For this reason we analyzed *zld's* expression in the developing larval wing discs. Alternatively spliced transcripts of *zld* encoding proteins lacking the 3 of 4 C-terminal zinc fingers, that were previously reported to be CNS specific variants [5], are shown here to be also produced in wing discs, together with the full length transcripts and a previously annotated transcript RC, that is not currently listed in FlyBase. Western blot analysis in wing discs and other larval tissue extracts, revealed the presence of two protein isoforms of about 180 and 70 kD. Their production is prominent in the imaginal wing discs.

Chromatin immunoprecipitation assays using larval wing discs revealed that Zelda binds to an intronic region of the *optomotor-blind* (*omb*) gene, a downstream target of the Dpp pathway which is involved in tissue growth and patterning processes in larvae [6–9].

Thus, the paradigm of Zelda function during embryogenesis where it coordinates the activity of many genes could also apply later in development, however more data would be necessary to support this hypothesis.

2. Materials and methods

2.1. Fly stocks

P{GawB}bi[md653] stock that expresses GAL4 in an *omb*[+] pattern was obtained from the Bloomington stock center. The *Zelda* RNAi stock (Transformant ID 38707) was obtained from the Vienna *Drosophila* RNAi center (VDRC). The full length open reading frame for Zelda was cloned into the *p*UAST vector and the UAS-Zelda

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0006-291X/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2013.07.071

stock was established after injecting the construct into w^{1118} embryos by the BestGene Inc (CA, USA). For the expression of the UAS constructs in the imaginal wing discs, flies carrying UAS-*zelda* and UAS-*zelda* RNAi were crossed to P{GawB}bi[md653] flies. The biological effects of these crosses were observed in the adult flies. *Oregon-R* was the wild type control. Flies were maintained on standard corn meal-agar media at 25° C.

2.2. Anti-Zld antibody

A mouse polyclonal antibody was raised against a chemically synthesized 14 a.a. peptide corresponding to the 460–473 a.a. region of ZLD protein and purified by Protein-A column, by GenScript (Piscataway, NJ, USA).

2.3. RNA extraction and RT-PCR (reverse transcription-PCR)

Total RNA was extracted from 30 wing discs or 15 L3 larvae with the TRIZOL reagent. The extracted RNA was further purified using the RNeasy Kit (Qiagen). 1 μg of total RNA was reverse transcribed using AMV RT (Finnzymes) and oligo(dT) primers in a final reaction volume of 20 μl. The PCR reactions were performed using 1 μl of cDNA per reaction mixture, 1.5 μM of each primer and 1U of DynaZyme polymerase (Finnzymes). The thermal cycle conditions consisted of an initial denaturation step and 35 cycles. (5 min 95° C and each cycle at 94° C for 30 s, 70° C for 30 s, 72° C for 1 min) followed by a final extension at 72° C for 10 min. The primer sequences used for detection of regions of the four annotated *zld* transcripts were:

RAf: 5′-CAGTGTGCATGTGTTTGTTTTTTTTCCG-3′,
RABCDr: 5′-TTGGAATGCCTCCCAGTCCG-3′
RABCDf: 5′-CATATGCACCGCTCTCGCCGTA-3′
RABCr: 5′-CGTGTGTTGTTCGTTCCTCCCG-3′
RDr: 5′-TCTGGGCGTTCGCTGGGTACAT-3′

It should be noted that the above primers do not amplify the full length of each transcript.

2.4. Total protein extracts from larval tissues – Western blotting

Wing discs and salivary glands dissected from L3 larvae as well as the remaining larval tissues were homogenized separately in cell lysis buffer containing 50 mM Tris–HCl pH 8.0, 150 mM NaCl, 1% NP-40, 125 μM DTT, 1 mM PMSF, 100 μg/ml Leupeptin (SIGMA), 20 μg/ml Aprotinin (SIGMA). Cellular debris were subsequently removed by centrifugation. A total 40 μg of protein extracts per lane were loaded on a 5% stacking – 10% resolving, SDS–polyacrylamide gel and transferred onto nitrocellulose membrane (Macherey–Nagel). Membranes were blocked in a PBT (1× PBS, 2% BSA, 0,1% Triton-X100) solution. Anti-ZLD antibody was used at 1:300. Horseradish peroxidase – conjugated goat anti-mouse antibody (Chemicon) was used at 1:4.000. The Western blot signals were detected using LumiSensor (GenScript).

2.5. Chromatin immunoprecipitation (ChIP) experiments

The ChIP protocol used is modified from Papantonis and Lecanidou [10] and Oktaba et al. [11]. Third instar *Oregon-R* larvae were dissected in ice-cold 1× PBS for a maximum of 20 min and guts and fat body were removed. Carcasses with discs attached were fixed for 20 min at RT by gently mixing in 1 ml cross linking solution. Cross linking reaction was stopped and carcasses remained in wash solution overnight at 4°C. 180 discs approximately were used per chromatin sample. The isolated wing discs, yielded 500–1000 bp chromatin fragments after sonication and subjected to ChIP using mouse anti-Zld and mouse anti-Frizzled (anti-Fz, DSHB) antibodies. Input and IP samples were subjected to low cycle PCR amplification with primers designed to amplify selected regions of the genes *decapentaplergic* (*dpp*), *engrailed* (*en*), *omb*, and *patched* (*ptc*), containing TAGteam related sequences. PCR products of amplified regions were cloned to a PCR 2.1 cloning vector (Invitrogen) and sequenced (Macrogen).

3. Results and discussion

3.1. Zelda affects growth of the developing wing

The primordium of *Drosophila* wing discs is established when a set of cells are set aside during embryogenesis and remain in a state of low transcriptional activity until larval development when they start to proliferate and differentiate extensively [12–14]. For this reason *Drosophila's* imaginal wing discs represent a model for the study and modeling of the developmental patterning and the interactions between signaling pathways that control tissue growth and differentiation. As already mentioned earlier, during embryonic development Zld functions as a major coordinator of gene networks required to promote MZT at earlier stages and of patterning decisions during later stages of embryogenesis [1–3]. Wing disc patterning could also require this type of control in order to establish the transcription of the proper set of genes that would promote development.

In order to study the functional role of Zelda in the imaginal wing discs development, we used the GAL4/UAS system, to express ectopically *zelda* in an *omb* pattern. The GAL4 system allows the selective expression of any gene construct inserted in a transgenic line, in a wide variety of tissue-specific patterns. A promoter or enhancer (GAL4 driver) directs expression of the yeast transcriptional activator GAL4 in a particular pattern and GAL4 in turn directs transcription of the UAS-target gene in the same pattern. When the GAL4 line is crossed to the UAS-target gene line, the target gene is turned on in the progeny [15,16].

The expression of *omb* occupies a central sector in the wing imaginal disc from second instar larval stage, when *omb* covers the whole presumptive wing domain, to third instar larval stage when its expression becomes restricted to the central most domain [8]. For this reason selection of the omb GAL4 driver is optimal for studying the possible effects of genes that are involved in wing growth processes. Ectopic expression of *zelda* in an *omb* pattern resulted in extensive loss of the adult wing tissue (Fig. 1), while expression of the *zelda* RNAi construct in the same region resulted in lethality during the pupal stage as the flies failed to eclose. These results showed that *zld* is essential for proper wing development, possibly affecting a number of genes that are necessary for this process.

3.2. Alternative spliced transcripts of zelda in larval tissues

It was already indicated by several previous studies, that *zld's* expression throughout embryogenesis shows a broad pattern during blastoderm formation while postblastoderm tissues that express *zld* are the developing CNS, the tracheal primordium and a set of midline neurons [1,4,5]. In FlyBase, *zelda* appears to have 3 transcripts RA (6610 nt), RB (7318 nt) and RD (7638 nt) and 3 annotated polypeptides PA (1596 a.a.), PB (1596 a.a.) and PD (1373 a.a.), respectively. The RC (5876 nt) transcript, listed previously in FlyBase, encodes a protein product PC (1367 a.a.) very similar to PD, their difference being in 6 a.a. Pearson et al. [5], studied the expression of the RB and RD transcript variants in the developing CNS, epidermis and imaginal disc primordia. Their study revealed that RB transcripts are reduced in the CNS, while the RD is present. On the contrary, the RD transcript was not detected in the imaginal disc primordia, where only the RB was present.

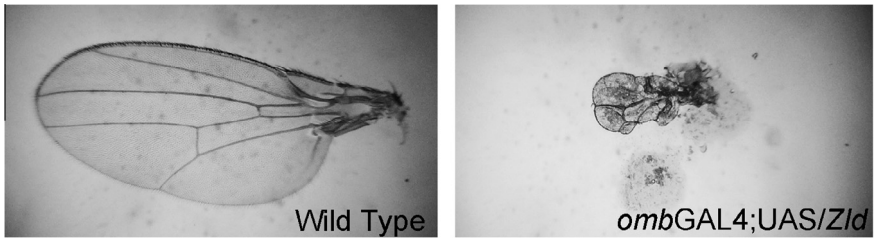

Fig. 1. *Drosophila's* adult wings. *Zelda's* ectopic expression induced by an *omb*GAL4 driver shows a defective phenotype with extensive tissue loss compared to the wild type strain.

In our study we designed specific primers that could amplify regions of all four annotated *zld* transcripts (Fig. 2A) and used them in PCR experiments with cDNA from *Drosophila* L3 larva wing discs as well as the rest of the remaining carcass which includes other imaginal discs and the CNS (Fig. 2B). The primer pair RAf and RABCDr was used to amplify the RA variant with an expected product size of 956 bp. RABCDf and RABCr primers were designed to recognize a region that corresponds to RA, RB, and RC transcripts. The expected PCR products are 2250 bp for both RA and RB variants while the RC variant, if present, would yield a product of 954 bp. RABCDf and RBCDr primers were used to amplify the RD transcript with an expected size of 609 bp. Staudt et al. [4], detected *zld* transcripts in mitotically active regions of the wing and eye imaginal discs in the larval stages of the fly. Our RT-PCR experiments revealed that transcripts RB, RC and RD are present in L3 wing discs while RA was not amplified (Fig. 2B). It should be noted though, that we detected RA transcript with the same pair of primers in pupae (Fig. S1), therefore its absence in larvae is not due to the PCR conditions e.g., primers. Interestingly, the RC transcript (954 bp), which is not currently listed in FlyBase, was expressed in the larval L3 stage, albeit we only detected it as a minor band in the isolated wing disc RNA. We therefore believe that RD is not an exclusively CNS transcript, since it is also present in the wing disc.

3.3. Zelda protein in larval tissues

In L3 larvae, the detected RB transcript variant is expected to be translated into the full length ZLD protein of 1596 a.a., while the RC and RD variants to 1367 and 1373 a.a. respectively, lacking the region coding for 3 of the 4 C-terminal zinc fingers. The theoretically deduced mass of these 3 protein products would be ~180 kD for PB and ~140 kD for PC and PD, respectively. Western blot experiments on total protein extract from L3 wing discs and the rest of the larval tissues using anti-Zld antibody, showed 2 bands estimated at ~180 kD, the expected MW for the protein coded by the RB transcript and one at ~70 kD. The intensity of both bands is reduced in the total larval extracts excluding the wing disc tissues (Fig. 3). Protein products around 140 kD that would correspond to the PC and PD isoforms of ZLD were not detected, instead a band at ~70 kD was detected. Protein extracts from larval salivary glands that were used as negative control, since *zelda* is not expressed in this tissue [17], gave no signal. Protein extracts from total larval tissues probed with preimmune serum showed no detectable signal as well (Fig. 3), therefore the band that appears at ~70 kD is not likely to be an unrelated product. Furthermore, the same two bands of ~180 and ~70 kD, are also detected in a Western Blot on total protein extracts from L3 larvae, using an antibody (rabbit anti-ZLD) raised against the 4-zinc finger region of ZLD protein (Fig. S2).

The above results suggest that a ZLD isoform (~70 kD) is prominently produced in the larval stage, which may be a specific proteolytic fragment of the protein (~140 kD) coded by the RC and RD transcripts, since the expression, especially, of the RD transcript is quite prominent in larvae (Fig. 2B). Alternatively, but less likely, this band could be the product of translation of a novel unknown *zld* transcript, although we failed to detect one with various combinations of primers in RT experiments. Thus, it is very likely, that this ~70 kD product could have a molecular function different from that of the PB polypeptide, an issue that is further investigated.

3.4. Chromatin Immunoprecipitation revealed Zelda's binding to an omb putative regulatory element

Our results from gain and loss of function experiments on the involvement of ZLD in the wing development, as well as other pre-

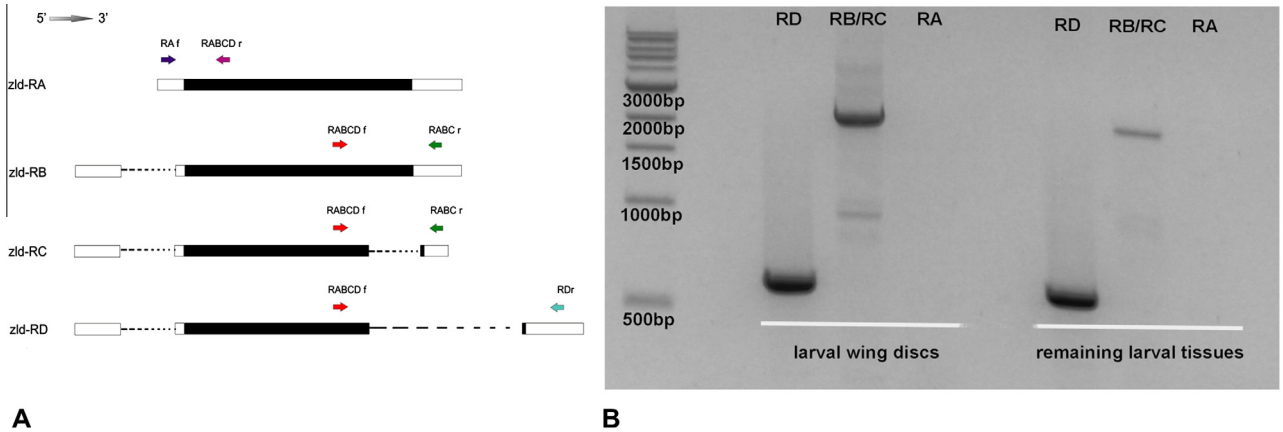

Fig. 2. *Zelda's* transcripts (A) and PCR products amplified from cDNA of larval wing discs and total larval tissues minus the wing discs (B). (A) Schematic representation of the annotated transcripts. The relative position of the primers used to specifically amplify *zld's* transcripts is shown by arrows. The transcript RC, not currently listed in the FlyBase, was detected in this study in tissues of the *Drosophila* larval stage. (B) The RB transcript appears at 2252 bp, the RC at 954 bp and the RD at 609 bp. The RA transcript was not detected in the larval stage. The RD transcript shows prominent expression in both cases, while RB and RC variants show lower amplification levels when the amplified cDNA from total larval tissues is compared to larval wing discs. Marker of the sizes is shown on the left.

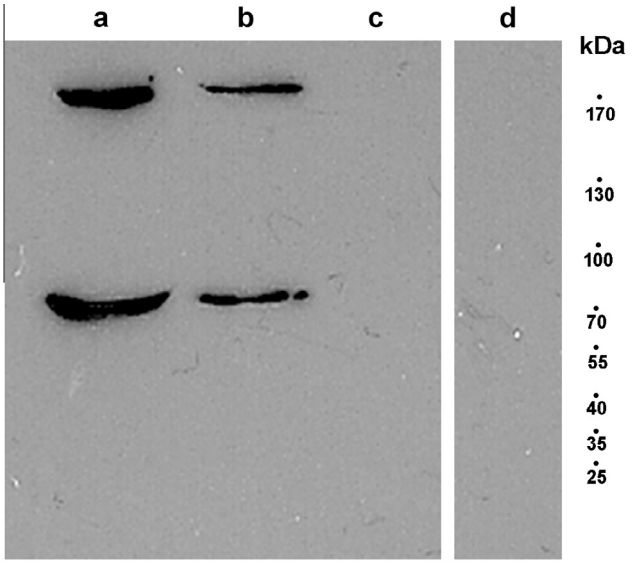

Fig. 3. Production of ZLD protein isoforms in larval tissues. For each lane, equal amounts (40 μg) of total protein extracts were separated on 10% SDS–PAGE and blotted onto nitrocellulose membrane. The blots were probed with mouse anti-ZLD at a dilution of 1:300. (A) Protein extracts from larval wing discs. 2 bands of ~180 and ~70 kD were detected. (B) Protein extracts from total larval tissues without the wing discs. The proteins detected show reduced production compared to wing disc protein extracts. (C) Protein extracts from larval salivary glands, used as negative control, since *Zelda* is not expressed in this tissue, gave no signal (see text). (D) Protein extracts from total larval tissues probed with pre-immune serum showed no detectable signal. The MW marker is shown on the right.

liminary results, led us to the hypothesis that ZLD could bind to genomic regions considered to be regulatory elements of genes essential for wing development. Liang et al. [1], using *zerknullt* (*zen*) enhancer sequences, have shown that in the embryo, ZLD binds with different affinities on TAGteam heptamer motifs. We have chosen two of the motifs found in *zen* enhancer (CTGCCTG and CTACCTG) and searched for their presence in the genomic regions of genes that we considered as probable candidates for regulation by ZLD. Selection of the regions was mainly based on data either supporting dense binding of transcription factors to chromatin or validated as putative regulatory elements [18]. The CTGCCTG sequence was found in a region ~1000 bp upstream of the 5′ UTR of omb-RD, omb-RC and omb-RE transcript variants of the *omb* gene and ~500 bp upstream of the 5′ UTR of the dpp-RA transcript. The CTACCTG sequence was found within a *patched* (*ptc*) intronic region ~2000 bp upstream its second exon, while a sequence of a putative enhancer of the *engrailed* (*en*) gene within its 5′ UTR of the en-RB transcript variant, lacking TAGteam heptamers, was chosen as a negative control.

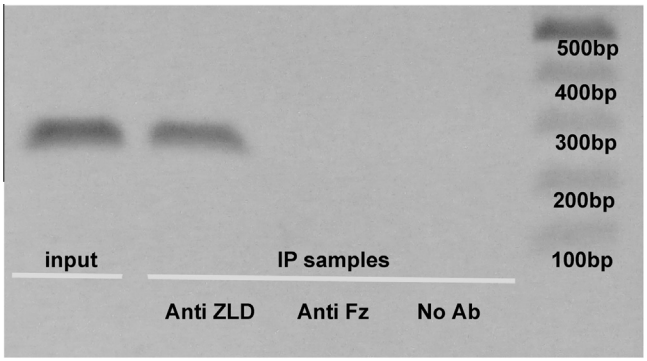

Fig. 4. ChIP assays on larval wing discs. The results display occupancy of an *omb* region by ZLD. IP samples were loaded on lanes 2, 3 and 4. Lane 2 represents a sample immunoprecipitated with the anti-ZLD antibody while lanes 3 and 4 correspond to negative controls, anti-Fz (Frizzled) and no antibody, respectively. Marker of the sizes is shown on the right.

Chromatin immunoprecipitation assays with the anti-Zld antibody, followed by amplification of the precipitated chromatin with specific primers designed for the above selected regions, with expected sizes for: *ptc* 349 bp, *omb* 233 bp, *dpp* 574 bp and *en* 314 bp showed amplification only for the *omb* locus (Fig. 4). The locus where we detected Zelda's binding belongs also to an intron of the *Dmel*/CG32773 gene, which however is not expressed in larval tissues as it is reported in FlyBase [17]. Within the amplified sequence (233 bp) and the overlapping adjacent sequences, recent findings report binding sites for a number of transcription factors such as Zinc finger homeodomain 1 (*zfh1*), Twist (*twi*), Senseless (*sens*), Disconnected (*disco*), Medea (*Med*) and others [18,19]. The exact role of these highly occupied transcription factor regions (HOT regions) remains obscure, however it was proposed that their putative functions could include a role in DNA replication and an interplay with boundary elements [19]. In a study of *Drosophila* embryonic development, Kvon et al. [20], reported that HOT regions, including motifs recognized by ZLD, can function as enhancers with diverse activity patterns.

Interaction of Zelda with *omb* indicates its involvement in the Dpp signaling pathway during wing disc growth. Dpp acts through binding to a receptor complex of Thickveins (*tkv*) and Punt (*put*) and it regulates expression of many target genes in a concentration dependent manner. The genes *spalt* (*sal*), *omb*, *Daughters against dpp* (*Dad*) and *vestigial* (*vg*) are activated in different distances from the Anterior/Posterior compartment border, while *brinker* (*brk*) is repressed [21]. The exact mechanism of *zld* action to a specific gene(s) in the Dpp pathway cannot be deduced by the data presented in this study. Moreover, the severe phenotypes observed by knocking down or overexpressing *zelda* using the omb GAL4 driver do not allow us to investigate the possibility of altered expression levels of the *omb* gene, where Zelda's binding was found, due to absence or lack of wing tissues after loss and gain of function experiments. However, our preliminary results using alternate GAL4 drivers in the imaginal wing discs show altered expression of the gene *vestigial* (*vg*), a Dpp target whose expression is Omb dependent [8], in the case of *zelda* knock down.

The observed ZLD's binding to the above region could be due either to a direct interaction with chromatin or through other(s) transcription factor(s). It is very likely though, that ZLD's binding to chromatin during larval development is not restricted to a small set of genes and leaves open the possibility of its involvement and in other signaling pathways.

Acknowledgments

We thank Drs. C. Delidakis and K. Iatrou for critical reading of the manuscript. This work was supported from the Special account of research grants of the University of Athens. P.G. was supported by the late Captain V. Constantacopoulos.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.07.071.

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