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Comparative gene expression analysis of *Dtg*, a novel target gene of Dpp signaling pathway in the early *Drosophila melanogaster* embryo

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ABSTRACT

In the early *Drosophila melanogaster* embryo, Dpp, a secreted molecule that belongs to the TGF-β superfamily of growth factors, activates a set of downstream genes to subdivide the dorsal region into amnioserosa and dorsal epidermis. Here, we examined the expression pattern and transcriptional regulation of *Dtg*, a new target gene of Dpp signaling pathway that is required for proper amnioserosa differentiation. We showed that the expression of *Dtg* was controlled by Dpp and characterized a 524-bp enhancer that mediated expression in the dorsal midline, as well as, in the differentiated amnioserosa in transgenic reporter embryos. This enhancer contained a highly conserved region of 48-bp in which bioinformatic predictions and *in vitro* assays identified three Mad binding motifs. Mutational analysis revealed that these three motifs were necessary for proper expression of a reporter gene in transgenic embryos, suggesting that short and highly conserved genomic sequences may be indicative of functional regulatory regions in *D. melanogaster* genes.

Dtg orthologs were not detected in basal lineages of Dipterans, which unlike *D. melanogaster* develop two extra-embryonic membranes, amnion and serosa, nevertheless *Dtg* orthologs were identified in the transcriptome of *Musca domestica*, in which dorsal ectoderm patterning leads to the formation of a single extra-embryonic membrane. These results suggest that *Dtg* was recruited as a new component of the network that controls dorsal ectoderm patterning in the lineage leading to higher Cyclorrhaphan flies, such as *D. melanogaster* and *M. domestica*.

1. Introduction

In the early *Drosophila melanogaster* embryo, Decapentaplegic (Dpp), the functional ortholog of vertebrates BMPs 2/4 forms a dorso-ventral (DV) signaling gradient that results in the subdivision of dorsal ectoderm into the presumptive dorsal epidermis and amnioserosa (Arora et al., 1994; Ashe et al., 2000; Ferguson and Anderson, 1992; Wharton et al., 1993), an extra-embryonic membrane that evolved in the lineage of higher Cyclorrhaphan flies, such as *D. melanogaster*, from two extraembryonic membranes, the amnion and the serosa, which are present in more basal flies (Rafiqi et al., 2008; Schmidt-Ott et al., 2010).

Even though Dpp acts as an inductive morphogen proper DV patterning requires the activity of Screw (Scw), another BMP homolog. Signaling of Dpp and Scw through Type I and Type II receptors leads to the phosphorylation of the Smad transcription factor, Mothers-against-dpp (Mad). Phosphorylated Mad forms a complex with a co-Smad, known as Medea, and both translocate into the nucleus to activate transcription of a number of downstream target genes, reviewed by Parker et al. (2004). Most of the genes identified as target of Dpp signaling pathway in the early embryo are required for amnioserosa development.

Abbreviations: *Dtg*, Dorsal target gene; Dpp, Decapentaplegic; TGF-β, Transforming growth factor beta; bp, base pair; *D. melanogaster*, *Drosophila melanogaster*; *M. domestica*, *Musca domestica*; BMP, Bone morphogenetic protein; DV, Dorso ventral; Scw, Screw; Mad, Mothers against decapentaplegic; *D. pseudoobscura*, *Drosophila pseudoobscura*; *D. virilis*, *Drosophila virilis*; NaCl, Sodium chloride; NaOCl, Sodium hypochlorite; $MgCl_2$, Magnesium chloride; EGTA, Ethylene glycol tetraacetic acid; DIG, Digoxigenin; FITC, Fluorescein isothiocyanate; GST, Glutathione S-transferase; MH1, Mad homology 1 domain; LB, Luria–Bertani (medium); PBS, Phosphate buffered saline; SDS-PAGE, Sodium dodecyl sulfate PA-gel electrophoresis; PCR, Polymerase chain reaction; MEME, Multiple em (expectation maximization) for motif elicitation; MAST, Motif Alignment and Search Tool; kb, kilobase; MLAGAN, Multiple limited area global alignment of nucleotides; MUSCLE, Multiple sequence comparison by log-expectation; TMHMM, Trans membrane hidden Markov model; pMad, Phosphorylated MAD; mRNA, Messenger ribonucleic acid; Tris, tris(hydroxymethyl) aminomethane; HCl, Hydrochloric acid; DTT, Dithiothreitol; KCl, Potassium chloride; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Zn2SO4, Zinc sulfate; CuSO4, Copper sulfate; PEG, Polyethylene glycol; EDTA, Ethylenediaminetetraacetic acid; KOAc, Potassium acetate; *D. simulans*, *Drosophila simulans*; *D. sechelia*, *Drosophila sechelia*; *D. erecta*, *Drosophila erecta*; *D. yakuba*, *Drosophila yakuba*; *D. ananassae*, *Drosophila ananassae*; *D. persimilis*, *Drosophila persimilis*; *D. willistoni*, *Drosophila willistoni*; *D. mojavensis*, *Drosophila mojavensis*; *D. grimshawi*, *Drosophila grimshawi*; Sog, Short gastrulation; GAL4, Yeast transcription activator protein; UAS, Upstream activation sequence; *lacZ*, β-galactosidase gen; Mya, Millions years ago.

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Among them, *zen*, a homeotic gene that is responsible of all aspects of amnioserosa differentiation (Rushlow and Levine, 1990) and the *u-shaped* group of genes, all encoding transcription factors involved in the maintenance of amnioserosa (Frank and Rushlow, 1996; Reim et al., 2003; Yip et al., 1997). Genes belonging to the *u-shaped* group share similar defective phenotypes that affect germ-band retraction and dorsal closure, two morphogenetic processes that depend on the integrity of amnioserosa (Schmidt-Ott, 2005).

Since the sequencing of the *D. melanogaster* genome, a series of high-throughput and reverse genetic methodologies have contributed to identify and characterize new genes functioning downstream of well-characterized signaling pathways (Furlong et al., 2001; Scuderi et al., 2006; Stathopoulos et al., 2002; Zúñiga et al., 2009). In a previous work, we used suppression subtractive hybridization and microarray analysis (Zúñiga et al., 2009) to isolate a set of transcripts that are differentially expressed between gastrulating and blastoderm embryos, among them we identified gene CG6234 and showed that it is specifically expressed along the dorsal midline of the early embryo and in the differentiated amnioserosa. Using an RNA interference knockdown strategy, we provided evidence that CG6234 gene product is required during germ-band retraction, a process that depends on the integrity of the amnioserosa tissue (Zúñiga et al., 2009).

Here, we report that the expression of gene CG6234, now named as *Dtg* (*Dpp target gene*), during dorsal ectoderm patterning depends on the Dpp signaling pathway. We identified a 524-bp enhancer located upstream of *Dtg* gene and demonstrate that three highly conserved Mad binding sites are necessary for expression of a reporter gene in the dorsal midline and amnioserosa of transgenic embryos. Thus, the analysis of *Dtg* enhancer suggested that short, highly conserved genomic sequences might be indicative of functional regulatory regions in *D. melanogaster* genes and that small changes within these sequences can alter the expression pattern of a gene.

Dtg orthologs with conserved expression patterns along the dorsal midline were detected outside Drosophilidae only in another higher Cyclorrhaphan fly, *Musca domestica*, suggesting that the origin of *Dtg* may correlate with the origin of a single extra-embryonic membrane. Taken together, our results indicate the existence of a new component that was incorporated within the network that controls dorsal ectoderm patterning in the lineage leading to higher Cyclorrhaphan flies.

2. Methods

2.1. Fly culture and embryo collection

D. melanogaster, *Drosophila pseudoobscura* and *Drosophila virilis* specimens were obtained from the Tucson *Drosophila* Species Stock Center and grown at 22 °C on standard cornmeal, molasses, agar, and yeast medium. Embryos were collected and staged as described in Zúñiga et al. (2009). Live larvae specimens of *Musca domestica* were acquired in Carolina Biological Supply Company and fed with an artificial wet diet based in milk, sugar and pellets of rabbit food, in a dark chamber at 26 °C. Adults were grown at 26 °C under 16 h L:8 h D and fed with a 1:1 mixture of granulated sugar and powder milk and moist wood shavings as water source. In order to stimulate fly ovoposition, Petri dishes containing wet cat food were introduced in adult's cages. Embryos were collected using a saline buffer (SB: 0.7% NaCl, 0.03% Triton X-100), dechorionized in 1:1 NaOCl:SB, washed and fixed for 1 h in 1:1 heptane and fixative solution (100 mM NaCl, 9.4% formaldehyde, 50 mM $MgCl_2$, 50 mM EGTA, 100 mM Tris pH 9, 0.1% Tween-20). Finally, embryos were washed three times in 100% methanol and 4 times in 100% ethanol and stored at −20 °C.

2.2. D. melanogaster strains

Canton-S flies were used as wild type strain. The alleles of mutant genotypes were: dpp^{H46} a null *dpp* allele balanced over *CyO23*, *P*[*dpp*+] due to the haploinsufficient nature of the *dpp* locus, heterozygous dpp^{H46}/+ flies are 95% lethal (St Johnston and Gelbart, 1987; Wharton et al., 1993), dpp^{hr92} a hypomorphic *dpp* allele balanced over *Cyo*, *ftz-lacB* (Wharton et al., 1993) and sog^{s6} balanced over *FM7*, *ftz-lacZ* (Hamaguchi et al., 2004). Homozygous mutant embryos were distinguished by the lack of *lacZ* mRNA detection in double *in situ* hybridization with a DIG-RNA probe. Flies carrying UAS-*dpp* have been described (FlyBase, http://flybase.bio.indiana.edu/), and they were crossed to a maternal Gal4 driver in which the Gal4 protein is expressed under the control of the maternal gene *nanos* promoter (P{GAL4-*nos*.NGT}40).

2.3. RNA probe preparation and in situ hybridization

DIG-RNA probes were prepared from a 335 bp gene fragment of *D. melanogaster Dtg* mRNA and a 607 bp gene fragment of *M. domestica Dtg*. *D. melanogaster Dtg* probe contained 72.3% of identical sites with a 79.7% of mean pair wise identity among *D. melanogaster*, *D. pseudoobscura* and *D. virilis* and it was used to analyze the expression pattern of *Dtg* in the different *Drosophila* species and strains. A plasmid bearing a *lacZ* insert (gift of Dr. M. Levine) was employed to prepare a RNA probe to detect the expression of the *lacZ* transgene. *In vitro* transcription was performed according to the manufacturer's instructions using Fluorescein (FITC)- or Digoxigenin (DIG)-RNA labeling mix (Roche, Mannheim, Germany) and the appropriate RNA polymerases. *In situ* hybridizations of *Drosophila* species and *M. domestica* were carried out essentially as described in Zúñiga et al. (2009). When needed, double *in situ* hybridizations of *D. melanogaster* embryos were performed using FITC- and DIG-labeled RNA probes, a sheep anti-DIG primary antibody (Roche) and a mouse anti-FITC primary antibody (Roche).

2.4. Immunostaining of embryos

Embryos were fixed and treated as described in Zúñiga et al. (2009). Primary antibodies were polyclonal anti-Phospho-smad 1/5 (Cell Signaling; 1:10) and monoclonal anti-Actin (Hybridoma Bank; 1:50), secondary antibodies were Alexa Fluor-488 goat anti-Rabbit IgG (Invitrogen; 1:500) and Cy3 donkey anti-mouse IgG (Jackson 1:500). Nuclear staining was made with ToPRO3 (Molecular Probes; 10 μM). Fluorescently-labeled embryos were mounted in Dabco–Mowiol solution. Confocal images were collected using the Confocal Laser Scanning Microscope-510 META (Zeiss) and processed using LSM Image Browser software (Zeiss) and Adobe Photoshop 7.0.

2.5. Expression and purification of recombinant Mad–GST protein

Expression plasmid encoding Mad–GST fusion protein containing the N-terminal MH1 domain was kindly donated by Professor Christine A. Rushlow (Rushlow et al., 2001). For expression of Mad–GST fusion protein, an overnight cultured *Escherichia coli* strain BL21 (Invitrogen) was inoculated into fresh LB medium, grown at 37 °C to an OD600 of 0.6 and induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 37 °C for 5 h with agitation. Cell pellets were harvested by centrifugation for 10 min at 3200 ×*g*, re-suspended and washed with cold PBS buffer including a protease inhibitor cocktail (Roche, Basel, Switzerland). Cell pellets were collected by centrifugation, re-suspended in 5 ml of cold lysis buffer (20 mM Tris–HCl, pH 7.5, 800 mM NaCl, 1 mM DTT, 0.5% Tween-20) for 15 min on ice and sonicated until lysis for 5 min at 45 s intervals on ice. The insoluble cell debris was removed by centrifugation for 30 min at 13,000 ×*g*. For purification of the recombinant proteins, the clarified supernatants were loaded onto columns containing glutathione–agarose (Sigma) under gravity flow. The resin was rinsed twice with PBS buffer and protein was eluted according to the manufacturer's instructions (General