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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

Electric Healthcare). Protein purity was verified by SDS-PAGE analysis and Coomassie Blue staining and quantified by the Bradford method.

2.6. Gel mobility shift assays

The following oligonucleotides were synthesized: a wild type 41 bp oligonucleotide that contains three predicted Mad binding sites and three mutant oligonucleotides containing different point mutations to abolish the predicted Mad binding sites. Wild-type and mutant oligonucleotides were denatured by heating and annealed at room temperature to a double-stranded DNA probe. Then, double strand oligonucleotides were end-labeled using 4 μl [γ-^{32}P] ATP (10 mCi/ml) in the presence of 10 units of T4 polynucleotide kinase (Promega) for 30 min at 37 °C and purified using a Nucleotide Removal Kit (Quiagen). Purified probes were incubated on ice with different concentrations of purified Mad–GST fusion protein for 30 min in KCl 40 mM, glycerol 12% and binding buffer (HEPES 30 mM, Zn_2SO_4 50 μM, DTT 5 mM, $CuSO_4$ 100 μM, $MgCl_2$ 1 mM, PEG-8000 2% and poly(dI)–poly(dC) 1 ng/μl). Reactions were then electrophoresed on a 5.6% non-denaturing polyacrylamide gel to constant voltage in 0.25× TBE buffer (Tris–HCl 1 M, boric acid 0.9 M and EDTA 0.01 M) using protocols described by Dent et al. (1999). Gel was dried for 2 h at 80 °C, exposed overnight to phosphor Imaging Screens-K (Kodak) and then scanned using the Personal Molecular Imager FX System (BioRad) and Quantity One Software (Kodak).

2.7. Reporter plasmid construction, site-directed mutagenesis and transgenesis

Genomic *D. melanogaster* DNA was prepared as described in Bellen et al. (2004) with minor modifications: homogenized samples were incubated at 70 °C and precipitated in ice for 30 min in the presence of a KOAc solution (5 M, pH 5.2). A phenol/chloroform extraction followed by ethanol precipitation was used in order to purify the DNA. DNA fragments encompassing nucleotides −804 to −409, −403 to +121 and −128 to +121 relative to the *Dtg* transcriptional start site were amplified by PCR. The forward primer sequences were as follows: 5′-GTAGCTGGGA CCGACG-3′ (map positions −128 to −112); 5′-CGGCGATCTTATCATTTC CCT-3′ (−804 to −783) and 5′-ATAGCCGGGCCAAAAAG-3′ (−403 to −386). Reverse primers were: 5′-CTCGAGACTTTCAGCTGTT-3′ (+102 to +121) and 5′-GACGCTGTGGATGTGAAGTG-3′ (−389 to −409). PCR products were purified and cloned into the pGEMT-Easy vector (Promega). Fragments were subcloned into the gypsy-insulated pPelican vector (Barolo et al., 2000). Enhancers were mutagenized in the pGEMT-Easy vector using the QuickChange Multi Site-directed Mutagenesis Kit (Stratagene). Constructs were sent to Genetic Services Inc. (Cambridge, MA) for production of transgenic flies. For each transgene, at least three independent insertions were isolated and analyzed.

2.8. Mad-binding sites prediction

In a series of previously known regulatory regions of Dpp target genes (training set), we identified overrepresented motifs using the software MEME (Bailey and Elkan, 1994). Search was performed on both strands with a maximum size of 13 bp. The position weight matrix (PWM) of each identified motif was converted to a logo representation in order to compare it with known transcription factors binding sites. Once a putative Mad PWM was identified in the training set, it was used to find potential Mad binding sites in the 524-bp Dtg3 enhancer using the MAST program with default options (Bailey and Gribskov, 1998). As a second bioinformatic approach, we applied a phylogenetic footprinting strategy (Janky and van Helden, 2008) to search for conserved motifs in 2 kb of the upstream non-coding region of *Dtg* orthologs. In doing so, *Dtg* orthologs from seven *Drosophila* species were aligned using the MLAGAN (Brudno et al., 2003) algorithm and the alignments were visualized with the VISTA Genome Browser (Frazer et al., 2004), conservation was measured in an 80 bp window with a cut-off score of 50% of identity in a row of 60 bp. Within these regions the BlockSampler program (Monsieurs et al., 2006) was used to identify potential Mad binding motifs. The nucleotide composition of intergenic regions of each genome was used to generate a background model. BlockSampler was executed using the default options of the program, except that motif size was 8 bp. The resulting motifs were sorted and ranked according to their information content using the MotifRanking program (Thijs et al., 2002). The fifteen best motifs were chosen to be compared with the results obtained by MEME/MAST.

2.9. Identification of Dtg orthologs and protein alignments

Orthologs for *D. melanogaster Dtg* protein were searched in the OrthoDB database (Waterhouse et al., 2011) and the sequences of 11 *Dtg* orthologs were collected from the FlyBase database. Accession identifiers are: *Drosophila simulans* GD18852, *Drosophila sechelia* GM24053, *Drosophila erecta* GG19127, *Drosophila yakuba* GE26211, *Drosophila ananassae* GF17112, *Drosophila persimilis* GL27146, *D. pseudoobscura* GA19462, *Drosophila willistoni* GK13722, *D. virilis* GJ14151, *Drosophila mojavensis* GI22850 and *Drosophila grimshawi* GH18703. Alignments of the twelve *Drosophila* protein sequences plus *M. domestica Dtg* were performed using MUSCLE software (Edgar, 2004) with 1000 iterations and neighbor joining option for clustering. Search for ortholog proteins in *Anopheles gambiae*, *Aedes aegypti*, *Culex quinquefasciatus*, *Megaselia abdita* and *Epysirphus balteatus* was performed using blastp alignments against available databases, and the following including criteria: E-value of 1×10^{-5}, a minimum of 35% of identity and alignment coverage >50%. Signal peptide prediction used SignalP (Petersen et al., 2011) and transmembrane helices prediction used TMHMM (Krogh et al., 2001).

2.10. Transcriptome sequencing an assembly

For RNA sequencing, we selected four early stages of *M. domestica* embryogenesis and collected 100 embryos from each one. RNAs were extracted using TRIzol reagent (Life Technologies) according to the manufacturer's instructions. Integrity of RNA samples was examined by electrophoresis on denaturing agarose gel. Analysis of absorbance using an Infinite 200 PRO Nanoquant (TECAN) spectrophotometer was used to quantify RNA mass and samples with a 260:280 ratio <2 were discarded. DNA was digested with Ambion Turbo DNAse and purified using the RNA clean up protocol from the RNAeasy Mini Kit (Quiagen). Messenger RNA was isolated from each sample using the MicroPoly(A) Purist Kit (Life Technologies) according to the manufacturer's instructions. An equal mass of mRNA from each stage was pooled and used for sequencing. Transcriptome sequencing was performed by Macrogen (Korea) in a Hiseq2000 platform (Illumina) with a 100 bp pair end library and 300 bp insert size. A total of ~50 millions of reads were generated and 93.4% of them were conserved after trimmed for quality using the fastx tool (publicly available at http://hannonlab.cshl.edu/fastx_toolkit/index.html). The high quality dataset was *de novo* assembled using Trinity software (Grabherr et al., 2011). As a result, 25,142 transcripts were produced with an average size of 606 bp (Study Accession Number: SRP026398). Functional annotation of the transcriptome was performed using Blast against the following protein databases: Uniprot, Swissprot, Genbank nr, TCDB, KEGG and PRIAM using an E-value of 1×10^{-10} as cut-off. Transcripts shorter than 200 bp after assembly were not considered for annotation.

3. Results and discussion

3.1. Dtg expression along dorsal midline depends on Dpp signaling

The pattern of *Dtg* expression in a dorsal longitudinal stripe of the early *D. melanogaster* embryo (Zúñiga et al., 2009) is similar to the expression pattern of other well-characterized Dpp target genes (Ashe et al., 2000), suggesting that *Dtg* expression may also be regulated by

Dpp signaling. Therefore, we examined whether there is a correlation between Dpp activity and *Dtg* expression during early stages of embryogenesis. To detect Dpp signaling activity we stained the wild-type embryos with an antibody that recognizes the phosphorylated and hence activated form of Mad (pMad, Fig. 1A). High levels of pMad were detected in a stripe of five to six dorsal cells, whereas cells at either side of the stripe showed low and undetectable levels of pMad. This is the expected expression pattern of pMad since a sharp, step gradient of pMad is formed as a consequence of peak levels of Dpp activity in dorsal cells (Dorfman and Shilo, 2001; Ross et al., 2001; Rushlow et al., 2001; Shimmi et al., 2005). As shown before (Zúñiga et al., 2009), *Dtg* mRNA was detected in a dorsal longitudinal stripe of variable width (4 to 14 cells) encompassing the developing amnioserosa and dorsal regions of the dorsal ectoderm, thus *Dtg* expression pattern seems to extend to areas that receive low Dpp inputs (Fig. 1B). In addition, faint *Dtg* signals extended into areas that lack detectable pMad (Fig. 1B, arrows). When *Dtg* expression pattern was analyzed in a *dpp* mutant background (Wharton et al., 1993), we observed that the longitudinal stripe of *Dtg* expression, but not the faint transverse stripe of *Dtg*, was absent in dpp^{hr92} homozygotes (Fig. 1C, arrows), revealing that the expression of *Dtg* requires Dpp signaling in the early embryo.

Previous works have proposed that the graded distribution pMad in the dorsal ectoderm results in the specification of three distinct thresholds of gene expression (Ashe et al., 2000). Thus, high, intermediate and low levels of pMad (Dorfman and Shilo, 2001; Rushlow et al., 2001) activate different sets of target genes to subdivide the embryo into domains of different developmental fates. As a consequence, target genes of the Dpp signaling pathway in the early *D. melanogaster* embryo are expressed in discrete domains along the dorsal midline and exhibit different widths of expression according to their sensitivity to Dpp signal (Ashe et al., 2000). For example, peak levels of Dpp signaling activate the expression of genes *Race*, *zerknüllt* (*zen*) and *hindsight* (*hnt*) in a stripe of 5–7 cells in the dorsal-most region of the embryo (Ashe et al., 2000; Rusch and Levine, 1997; Tatei et al., 1995), whereas high but not maximum levels of Dpp signaling are required to activate the expression of *tailup* (*tup*), *u-shaped* (*ush*) and *C15* genes in a wider stripe of 12–14 cells (Ashe et al., 2000; Lin et al., 2006). Finally, the expression of *pannier* extends to into lateral regions with low to undetectable levels of pMad staining (Jazwinska et al., 1999). In the case of gene *Dtg*, its dorsal longitudinal stripe of expression is similar to that of genes *tup* and *ush* (Ashe et al., 2000), suggesting that *Dtg* requires high, but not peak, levels of Dpp signaling for activation.

Sog, a morphogen that acts primarily as an antagonist of Dpp and Scw, is required to ensure peak levels of Dpp signaling in the early embryo (Ashe and Levine, 1999; Podos and Ferguson, 1999). Thus, in sog^{S6} mutant embryos, pMad does not accumulate in the dorsal five to six cells, but instead pMad is present at in a broad dorsal domain (Dorfman and Shilo, 2001). We observed that in sog^{S6} embryos, *Dtg* expression on the dorsal midline domain became broadened (Fig. 1D), supporting the idea that high but not peak levels of pMad are required for *Dtg* expression in the early embryo. We also examined the effects of mutating *brinker* (*brk*), which encodes a transcriptional repressor that is a component of the Dpp signaling pathway in the *D. melanogaster* embryo (Jazwinska et al., 1999). We observed that *Dtg* expression was not affected in a *brk* mutant embryo (data not shown). Finally, when we examined the expression of pMad and *Dtg* in an embryo that overexpressed Dpp by using maternally expressed GAL4 to drive expression of UAS-*dpp* (*nos*-Gal4 > UAS-*dpp*), we observed wider dorsal longitudinal stripes of both pMad (Fig. 1E) and *Dtg* (Fig. 1F) expressions. Taken together, these observations suggested that proper *Dtg* expression along the dorsal midline of early *D. melanogaster* embryos requires Dpp signaling. *Dtg* requirement of high but not peak levels of pMad for expression on dorsal domain is similar to that of the *u-shaped* group of genes, which are implicated in the maintenance of the amnioserosa during embryogenesis. Moreover, defects in the expression of genes as *tup* and *ush* result in an embryo with several alterations in three morphogenetic processes required a normal amnioserosa tissue: germ-band retraction, head involution and dorsal closure (Frank and Rushlow, 1996). These phenotypic alterations are similar to the defects caused by RNAi against *Dtg* as it was previously reported in Zúñiga et al. (2009). According to these data, *Dtg* seems to be a new member of the genetic network involved in amnioserosa differentiation under the control of the Dpp signaling pathway.

3.2. Dtg is a target gene of Dpp signaling pathway

In order to characterize the regulatory regions of *Dtg*, the expression of three *lacZ* reporter constructs carrying different segments of the upstream non-coding region of *Dtg* was analyzed in transgenic flies. This resulted in the isolation of a 524 bp fragment, spanning nucleotides −403 to +120 relative to the transcription start site of *Dtg* (Supplementary data), named as Dtg3 enhancer, which drove *lacZ* expression in the mid-dorsal region of a stage 6 embryo in a pattern that was similar to that of the endogenous gene (Fig. 2A). The Dtg3 enhancer also drove

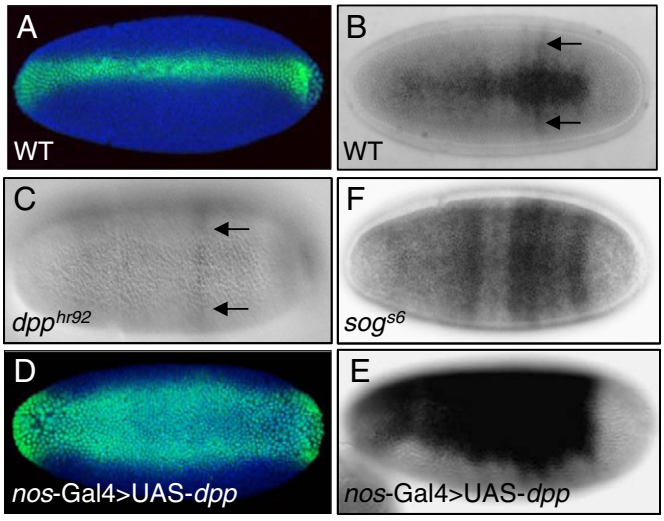

Fig. 1. Expression of *Dtg* in mutant backgrounds. (A–F) Dorsal views of late stage 5/early stage 6 embryos with anterior to the left. (A) Wild-type (WT) embryo stained with an anti-phospho-Smad1/5 antibody, which recognizes phosphorylated Mad (pMad, green) and the DNA probe ToPRO3 (blue). (B and C) Wild-type and mutant dpp^{hr92} embryos hybridized with a *Dtg* probe. (D) Mutant sog^{s6} embryo hybridized with a *Dtg* probe. (E) *nos*-Gal4 > UAS-*dpp* embryo stained with an antiphospho-Smad1/5 antibody (green) and ToPRO3 (blue). (F) *nos*-Gal4 > UAS-*dpp* embryo hybridized with a *Dtg* probe.

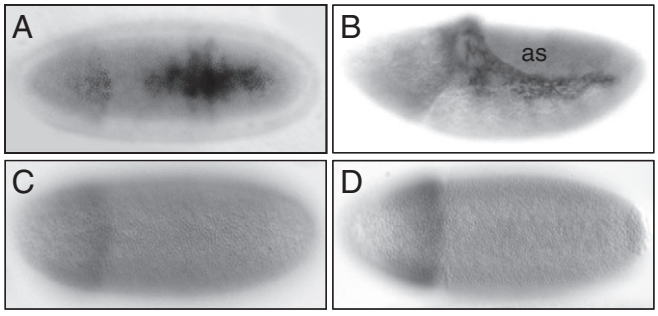

Fig. 2. A 540-bp enhancer drives *Dtg* expression. **(A)** Transgenic embryos of stage 6 and **(B)** stage 9 carrying the Dtg3-*lacZ* construct were *in situ* hybridized with *lacZ* probes. **(C)** Expression of Dtg3-*lacZ* was undetectable in dpp^{H46} heterozygous embryos. **(D)** In an embryo carrying mutations in the three conserved Mad-binding sites (Dtg3m3-*lacZ*), *lacZ* expression is severely reduced. The ring of staining in the head region is an artifact of the vector. Embryos are oriented with the anterior region to the left. A, C and D are dorsal views, B is a lateral view.

strong *lacZ* expression in the differentiated amnioserosa in stage 9 embryos (Fig. 2B). As expected, expression of *lacZ* was lost in dpp^{H46} heterozygote embryos (Fig. 2C), suggesting that similar regulatory mechanisms apply for Dtg3 enhancer as for the *Dtg* gene. Thus, these results indicate that the 524 bp fragment directed expression and mediated Dpp responsiveness in embryo domains and developmental stages that were comparable with the endogenous gene.

In *D. melanogaster*, Mad binding sites contain repeats of the degenerate sequence GNCN, which is consistent with the sequence of the Smad binding element (SBE), GTCT, found in the response regions of TGFβ target genes (Shi et al., 1998; Zawel et al., 1998). In addition, the sequence GRCGNC has been shown to recruit Mad proteins in *D. melanogaster* (Gao et al., 2005; Pyrowolakis et al., 2004). In this work, we used two bioinformatic approaches (see Methods section) to predict potential Mad binding sites within the Dtg3 enhancer. By using the MEME/MAST programs several CG-rich motifs were identified in the 524-bp sequence of Dtg3. Then, to improve the accuracy in the computational predictions of Mad binding sites, we applied a second strategy, known as phylogenetic footprinting, to detect conserved motifs in non-coding regions of *Drosophila* species. In doing so, we compared 2 kb of the non-coding region of *Dtg* ortholog sequences in eight species of *Drosophila* and visualized the alignments using the VISTA Genome Browser (Supplementary data). We detected a highly conserved region of 46-bp that is part of the Dtg3 enhancer and contains three potential Mad binding motifs (SBE motif: GNCN). These binding sites that were predicted by both MEME/MAST and BlockSampler programs were highly conserved in the eight *Drosophila* species examined (Supplementary data).

To examine whether Mad acts as a direct regulator on the *Dtg* enhancer, we performed *in vitro* DNA-binding experiments (Fig. 3). Our results from gel mobility shift assays showed that an oligonucleotide containing the three conserved Mad binding motifs predicted within the Dtg3 enhancer (Fig. 3A, letters in bold) was efficiently bound and shifted by recombinant Mad protein (Fig. 3B lanes 2–5). The relative contribution of each Mad binding motif to GST-Mad binding was analyzed by introducing point mutations into the wild type oligonucleotide. Mutations in one and two Mad conserved motifs in oligonucleotides m1 and m2 (Fig. 3A) reduced binding to Mad (Fig. 3C, lanes 4–7 and 9–12), however, only when the three conserved sites were mutated (Fig. 3A, m3) binding to Mad was abolished (Fig. 3C, lanes 14–17).

Based on the results obtained by gel mobility shift assays, mutations on the three conserved Mad binding motifs were engineered into the Dtg3 enhancer (Dtg3m3-*lacZ*), which was tested by analyzing the expression of the *lacZ* reporter gene in transgenic flies (Fig. 2D). We observed that *lacZ* expression was undetectable in embryos carrying the mutations, indicating the importance of this cluster of Mad binding motifs for the transcriptional activity of *Dtg*. Thus, we identified a small non-coding region that can drive high levels of transcription in the dorsal midline region and the differentiated amnioserosa of *D. melanogaster* embryos. This region contains a segment in which 86% of the nucleotides are conserved within all *Drosophila* species examined. These results demonstrate that *Dtg* gene contains short and highly conserved genomic signatures with regulatory function. Within the conserved regulatory region of *Dtg*, three Mad binding sites are required for its transcriptional activation in the presumptive amnioserosa region.

Other known Dpp targets are regulated by proximal enhancers that contain functional sites for the binding of Mad transcription factor. These regulatory regions have been characterized for genes expressed during early embryogenesis in distinct threshold concentrations of pMad gradient (Raftery and Sutherland, 2003). The results from these studies have supported the idea that Mad interacts with other transcription factors to induce tissue specific expression of Dpp target genes. As an example, proper expression of gene *Race* requires the binding of both Mad and Zen to its enhancer (Xu et al., 2005). In the case of *zen* and other Dpp target genes, activation depends on a concentration-dependent competition between Mad and the negative regulator Brk for overlapping binding sites within their enhancers (Jazwinska et al., 1999; Kirkpatrick et al., 2001; Rushlow et al., 2001). Because expression pattern of *Dtg* was not affected in *brk* mutant embryos, *Dtg* activation

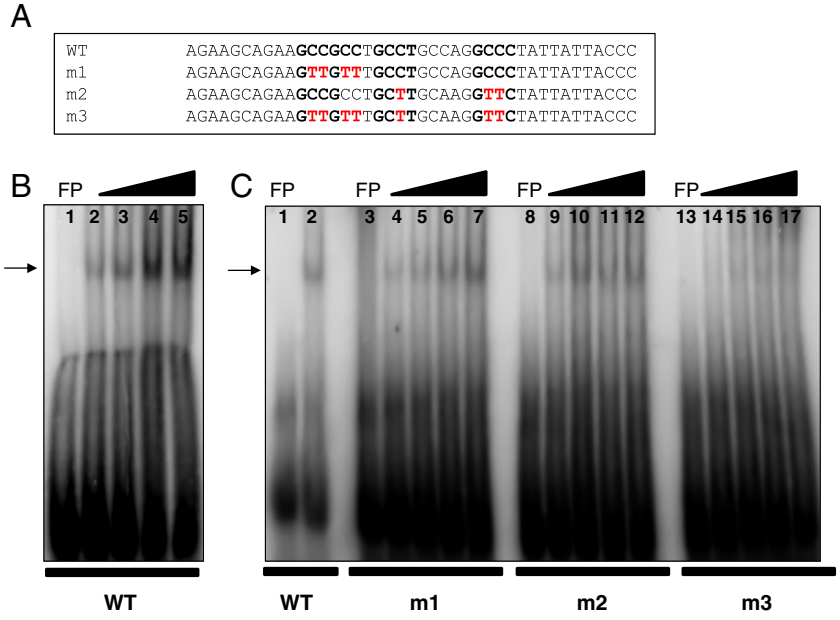

Fig. 3. Mad binding to *Dtg* enhancer. **(A)** The sequences of the Mad binding sites tested in the gel mobility shift assays are shown in bold with mutations highlighted in red. **(B)** Gel mobility shift assays of GST–Mad incubated with wild-type oligonucleotide. The first lane contains free probe. Lanes 2 to 5 contain increasing amounts of GST–Mad (50, 150, 250 and 500 ng). **(C)** GST–Mad (150 ng) was incubated with wild-type (lane 2) or mutated oligonucleotides. Lanes 4–7, mutations in one of the conserved sites. Lanes 9–12, mutations in two of the conserved sites. Lanes 14–17, mutations in the three conserved sites. Lanes contain increasing amounts of GST–Mad (50, 150, 250 and 500 ng). Lanes 1, 3, 8 and 13 contain free probe.

might rely on Mad proteins or, alternatively, might require the recruitment of a still unknown co-activator.

3.3. Cross species conservation of Dtg

To identify orthologs of *D. melanogaster Dtg*, the predicted *Dtg* protein sequence was used to query the genomes of twelve *Drosophila* species as well as the available genomes from the mosquitoes *A. gambiae*, *Culex quinquefasciatus* and *A. aegypti* (Arensburger et al., 2010; Holt et al., 2002; Nene et al., 2007). This search revealed the presence of *Dtg* orthologs in the 11 species of the *Drosophila* genus; however the mosquito genomes seemed to lack *Dtg* orthologs based on the criteria (E-value, identity and coverage) that were described in the Methods section. Comparison of the deduced amino acid sequences of *Dtg* orthologs in *Drosophila* species revealed a mean pair wise percent identity of 60.1% (Fig. 4). Protein sequence homology between the distant sibling species *D. melanogaster* and *D. grimshawi* was low (45.3%), except for the general organization of the protein. Conserved features among these sequences included a putative transmembrane domain located between amino acids 633 and 655 (88.5% of sequence identity) and a predicted signal peptide domain within the first 60 protein residues (54.6% of sequence identity).

The lack of *Dtg* orhologs in more distant mosquito genomes suggests that *Dtg* might represent an innovation of higher Diptera (Cyclorrhaphan flies), recently incorporated into Dpp signaling network. In order to explore this possibility, we extended our search for *Dtg* orthologs to the dipterans species *M. abdita* and *E. balteatus* for which transcriptome data is available through the Diptex database (Jimenez-Guri et al., 2013). However, using our ortholog identification protocol, we failed to detect the presence of Dtg sequences in these fly transcriptomes. Given that both *M. abdita* and *E. balteatus* are Cyclorrhaphan flies but, unlike *Drosophila*, belong to basal branches of this taxon (lower Cyclorrhaphan), we decided to examine whether *Dtg* orthologs were present in the transcriptome of *M. domestica*, a higher Cyclorrhaphan fly.

Even though *D. melanogaster* and *M. domestica* are evolutionary separated by at least 100 million years (Hennig and Pont, 1981), the morphology and early embryology of these higher Cyclorrhaphan flies are very similar (Weismann, 1864). In particular, in *M. domestica*, as well as in *Drosophila* species, dorsal ectoderm patterning leads to the formation of a single extra-embryonic membrane, the amnioserosa that covers only the dorsal region of the embryo, whereas in lower Cyclorrhaphan, two extra-embryonic membranes are present: the amnion which is restricted to the dorsal side of the embryo and the serosa that expands to ventral embryonic regions (Lemke and Schmidt-Ott, 2009; Rafiqi et al., 2008; Schmidt-Ott et al., 2010). Using a library of transcripts generated by assembling the raw data produced by sequencing the RNA of early developmental stages of *M. domestica*, we were able to identify a *M. domestica Dtg* transcript of 2352 bp which was translated into a protein of 783 residues. Comparison between the deduced amino acid sequences of *M. domestica* and *D. melanogaster Dtg* revealed a pair wise sequence identity of 27.7%. The overall structure of *D. melanogaster Dtg* is conserved in *M. domestica*. As with the *D. melanogaster*, *M. domestica Dtg* has a 60-residue long signal peptide followed by an extracellular domain, a transmembrane domain with a 61.5% of identity to the domain predicted in *D. melanogaster*, and a short cytoplasmic domain (Fig. 4).

Then, we sought to determine whether the gene expression pattern of *Dtg* in dorsal embryonic domains is conserved among *Drosophila* species and *M. domestica*. With this aim, *D. melanogaster*, *D. pseudoobscura* and *D. virilis* embryos, which we selected as examples, were probed with a labeled *Dtg* specific probe isolated from *D. melanogaster* (for details see the Methods section) (Fig. 5). In all of them, *Dtg* transcripts were first detected in stage 5 embryos (cellular blastoderm stage; Fig. 5, panels 1–4) in a dorsal longitudinal stripe. During gastrulation (Fig. 5, panels 5–8) *Dtg* mRNA staining was detected in the cells of the presumptive amnioserosa (asterisks). At later stages of embryogenesis (Fig. 5, panels 9–16), *Dtg* continues to be expressed in the dorsal region and it can be clearly observed in the differentiated amnioserosa tissue (as). The similar spatial and temporal expression patterns of *Dtg* in distantly related Diptera suggest that regulatory mechanisms for its expression during embryogenesis might be also conserved in these species. Accordingly, pMad the main effector of the Dpp pathway was also detected in embryos of *Drosophila* species and *M. domestica* (Fig. 5, panels 17–20).

Taken together, these results suggest that the dorsal expression pattern of *Dtg* during embryogenesis is conserved in higher Cyclorrhaphan flies, suggesting that the reported role of *Dtg* in amnioserosa maintenance might be also conserved. Moreover, *Dtg* origin could be placed 100 Mya, before the *Drosophila* radiation, since amnioserosa origin has been suggested to occur between 85 and 145 Mya (Rafiqi et al., 2010), *Dtg* orthologs, might have been incorporated into Dpp signaling network concomitant with the differentiation of a single extra-embryonic membrane.

4. Conclusions

Dpp signaling pathway is central to patterning in the dorsal region of the embryo, however few downstream target genes are currently known for the Dpp/Mad pathway, and thus the existing information is not enough to explain the regulatory network underlying the complex process of Dpp-dependent dorsal fate specification. In this work we present a new target gene of the Dpp pathway, *Dtg*, which encodes a putative transmembrane protein with roles in amnioserosa maintenance. Thus, our finding that *Dtg* is a downstream target of the Dpp signaling pathway reveals a link between dorsal ectoderm patterning and cellular

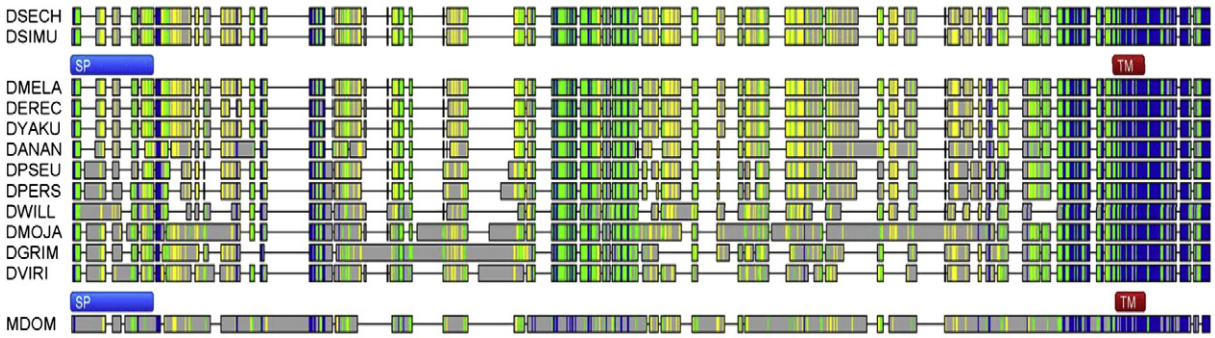

Fig. 4. Sequence alignment of *Dtg* protein across three *Drosophila* species and *M. domestica*. Sequences of *Dtg* orthologs were aligned using MUSCLE software. For each column in the alignment colors represents the similarity of the residues, which is based on the values from Blosum 62 substitution matrix: blue for 100% similar residues, green for over 80% of similarity, yellow for 60%–80% similarity and gray for similarity below 60%. Residues for signal peptide (SP) and transmembrane domain (TM) are depicted with blue and red bars, respectively, over *D. melanogaster* and *M. domestica* sequences.

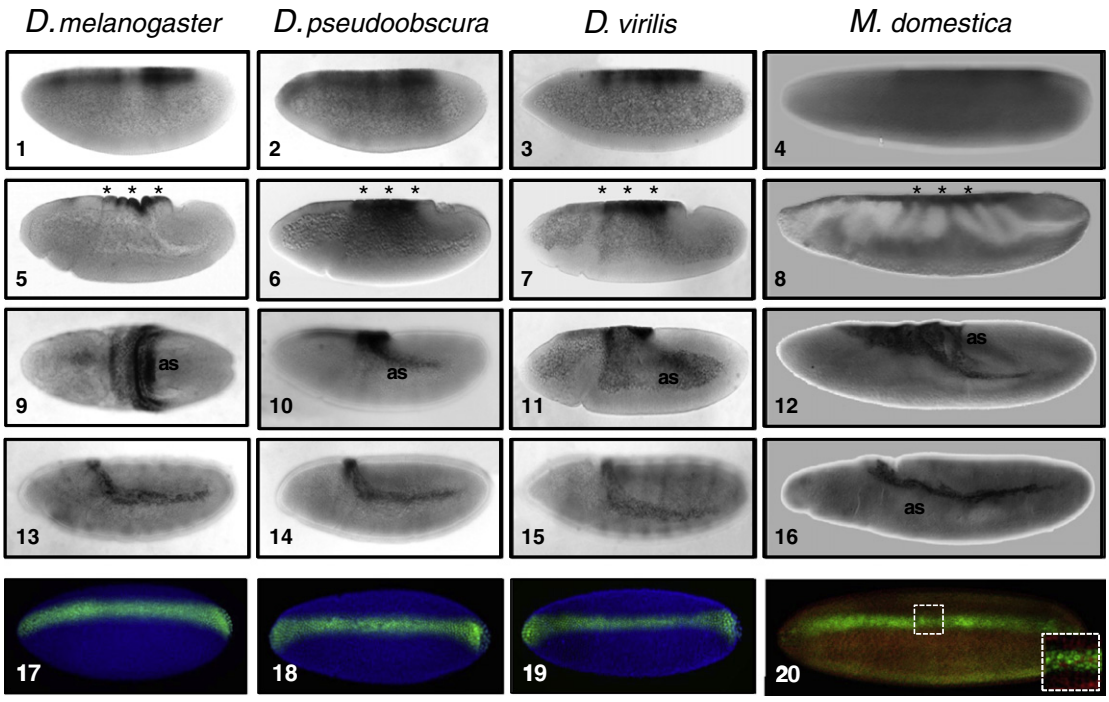

Fig. 5. Comparison of the *Dtg* expression pattern among three *Drosophila* species and *M. domestica*. **(Panels 1–16)** distribution of *Dtg* mRNA in embryos of stage 5 (1–4), stage 7 (5–8), stage 8 (9–12) and stages 9–12 (13–16). Embryos are oriented with the anterior region to the left. **(Panels 17–19)** stage 5 embryos from *Drosophila* species were stained with anti-phospho-Smad1/5 antibody (green) and ToPRO3 (blue). **(Panel 20)** *M. domestica* embryo (stage 5) was stained with anti-phospho-Smad1/5 antibody (green) and anti-actin (red), the inset is a higher magnification of the dorsal embryonic cells expressing pMad. All are lateral views of embryos, except panels 9 and 17–20 that are dorsal views.

functions involved in amnioserosa differentiation. In addition, the evidence presented here indicates an evolutionary conservation of sequence and expression of *Dtg* in higher Cyclorrhaphan flies and suggests a conserved role in amnioserosa maintenance.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.gene.2013.11.032.

Conflict of interest statement

The authors declare that there is no conflict of interest.

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