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Research paper

Target genes of Dpp/BMP signaling pathway revealed by transcriptome profiling in the early *D. melanogaster* embryo

Calixto Dominguez [a,1], Alejandro Zuñiga [a], Patricia Hanna [a,b], Christian Hodar [a,b], Mauricio Gonzalez [a,b], Verónica Cambiazo [a,b,*]

[a] Laboratorio de Bioinformática y Expresión Génica, INTA-Universidad de Chile, El Libano 5524, Santiago, Chile
[b] Fondap Center for Genome Regulation (CGR), Chile

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ABSTRACT

In the early *Drosophila melanogaster* embryo, the gene regulatory network controlled by Dpp signaling is involved in the subdivision of dorsal ectoderm into the presumptive dorsal epidermis and amnioserosa. In this work, we aimed to identify new Dpp downstream targets involved in dorsal ectoderm patterning. We used oligonucleotide *D. melanogaster* microarrays to identify the set of genes that are differential expressed between wild type embryos and embryos that overexpress Dpp (*nos*-Gal4>UAS-*dpp*) during early stages of embryo development. By using this approach, we identified 358 genes whose relative abundance significantly increased in response to Dpp overexpression. Among them, we found the entire set of known Dpp target genes that function in dorsal ectoderm patterning (*zen*, *doc*, *hnt*, *pnr*, *ush*, *tup*, and others) in addition to several up-regulated genes of unknown functions. Spatial expression pattern of up-regulated genes in response to Dpp overexpression as well as their opposing transcriptional responses to Dpp loss- and gain-of-function indicated that they are new candidate target genes of Dpp signaling pathway. We further analyse one of the candidate genes, CG13653, which is expressed at the dorsal-most cells of the embryo during a restricted period of time. CG13653 orthologs were not detected in basal lineages of Dipterans, which unlike *D. melanogaster* develop two extra-embryonic membranes, amnion and serosa. We characterized the enhancer region of CG13653 and revealed that CG13653 is directly regulated by Dpp signaling pathway.

1. Introduction

It is now well established that the graded concentration of a signaling molecule, known as morphogen, organizes and patterns tissues in developing animals (Wolpert, 1996). Studies in *Drosophila melanogaster* and in vertebrates have revealed that extracellular activity gradients of morphogens, such as members of the Hedgehog, Wingless and TGFβ families of signaling molecules, regulate the expression of target genes in a concentration-dependent manner (Raftery and Sutherland, 2003). In the early *D. melanogaster* embryo, the combined actions of two morphogens, Decapentaplegic (Dpp), the *Drosophila* functional ortholog of mammalian BMP2/4, and Screw (Scw) control the subdivision of dorsal ectoderm into presumptive dorsal epidermis and amnioserosa, an extraembryonic membrane that develops at the dorsal-most region of the embryo. The amnioserosa is found in higher cyclorrhaphan flies, such as *D. melanogaster* however in other dipterans, dorsal ectoderm patterning gives rise to distinct serosal and amniotic epithelia (Rafiqi et al., 2008; Schmidt-Ott et al., 2010).

During dorsal ectoderm patterning, Dpp and Scw form an extracellular gradient with peak levels of signaling at the dorsal-most region of the embryo (Raftery and Sutherland, 2003). The shaping of the Dpp gradient from an initially uniformly distributed mRNA is achieved by the combined action of extracellular Dpp binding proteins and metalloproteases (Matsuda et al., 2016). In the early embryo, Dpp acts as an inductive morphogen; however Scw enhances the pathway activity along the dorsal midline and is required for amnioserosa specification (Arora et al., 1994). Dpp/Scw signal trough Type I and Type II receptors leading to the phosphorylation of the Smad transcription factor, Mothers-against-dpp (Mad). Phosphorylated Mad (pMad) forms a complex with a co-Smad, known as Medea (Med), and both translocate into the nucleus to activate transcription of an undetermined number of target genes (Parker et al., 2004). Within the regulatory regions of known target genes, Mad/Medea bind to sites containing repeats of the degenerate sequence GNCN, which is consistent with the sequence of the Smad

Abbreviations: DIG, digoxigenin; FITC, fluorescein isothiocyanate; DAPI, 4′,6-diamino-2-fenilindol; PBS, phosphate-buffered saline; Cy3, cyanine 3; Cy5, cyanine 5; PCR, polymerase chain reaction.

* Corresponding author at: Laboratorio de Bioinformática y Expresión Génica, INTA-Universidad de Chile, El Libano 5524, Santiago, Chile.
E-mail address: vcambiaz@inta.uchile.cl (V. Cambiazo).
[1] Current address: Computational Biology Lab, Center for Bioinformatics and Genome Biology, Fundacion Ciencia y Vida. Zañartu 1482, Santiago, Chile.

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binding element (SBE) GTCT found in the response regions of TGFβ target genes (ten Dijke et al., 2000).

Most of the known targets of Dpp/Scw signaling are required for amnioserosa development. For example, *zen*, a homeotic gene that is responsible of all aspects of amnioserosa differentiation (Rushlow and Arora, 1990) and the *u-shaped* group of genes that encode transcription factors involved in the maintenance of amnioserosa once it has been differentiated (Frank and Rushlow, 1996; Yip et al., 1997; Reim et al., 2003). Recently, it has been shown that proper formation of Dpp gradient in the early embryo depends on a feedback regulation provided by the products of two target genes of Dpp pathway, *eiger* and *crossvein-2*, which stimulate and antagonize Dpp signaling, respectively (Wang et al., 2008; Gavin-Smyth et al., 2013).

In a previous work, we identified *Dtg*, a new target gene of Dpp signaling, which encodes a novel secreted protein with roles in amnioserosa maintenance (Zúñiga et al., 2009; Hodar et al., 2014). Here, we aimed to identify new Dpp downstream targets involved in dorsal ectoderm patterning. To do this, we used microarray transcriptome profiling, which enabled us to find direct and indirect transcriptional targets, including those which are difficult to identify in traditional mutant screens due to pleiotropy and/or functional redundancy. In addition, we further characterize gene CG13653 whose expression at the dorsal-most region of the early embryo was directly controlled by Dpp signaling.

2. Methods

2.1. Fly culture and embryo selection

Adults were grown at 22 °C on standard cornmeal, molasses, agar and yeast medium. Embryos were collected as described in Zúñiga et al. (2009). Flies carrying UAS-*dpp* have been described (FlyBase ID: FBst0001486), they were crossed to a Gal4 driver in which the Gal4 protein is expressed under the control of the enhancer of the maternal gene *nanos* (FlyBase ID: FBst0004442). In these embryos, induction of ectopic Dpp results in a broader longitudinal stripe of nuclear pMad when compared with wild type embryos (Hodar et al., 2014). In control embryos expression of *lacZ* was driven by *nanos*-Gal4 (*nos*-Gal4>*lacZ*). The alleles of mutant genotypes were: dpp^{hr92} a hypomorphic *dpp* allele balanced over *Cyo*, *ftz-lacB* (Wharton et al., 1993), dpp^{H46} a null *dpp* allele balanced over *CyO23*, P[*dpp* +] (Wharton et al., 1993), sog^{s6} balanced over FM7, *ftz-lacZ* (Hamaguchi et al., 2004) and brk^{M68} balanced over FM7, *ftz-lacZ* (Weiss et al., 2010). Homozygous mutant embryos were distinguished by the lack of *lacZ* mRNA detection in double *in situ* hybridizations. To obtain staged embryos, females were allowed to lay eggs for 2 h on 2% apple juice agar plates spread with live brewer's yeast. The plates were replaced several times and finally one-hour embryos were collected, dechorionated and washed with Ringer *Drosophila* solution (182 mM KCl, 46 mM NaCl, 3 mM CaCl2, 10 mM Tris-HCl, pH 7,2). Embryos were selected at stages 2–3 (syncytial blastoderm) or 5 (cellular blastoderm) and then allowed to continue their development in a humidified chamber at 25 °C. We hand-selected embryos at late stage 5 to stage 7 based on their morphological characters (Campos-Ortega and Hartenstein, 1985), and rapidly frozen them in liquid N_2. Embryos were kept at −80 °C for 1–2 weeks.

2.2. In situ hybridization of whole-mount embryos

In situ hybridizations using 1–2 ng/μL DIG-labelled RNA probes were carried out essentially as described in Hodar et al. (2014). 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. Double *in situ* hybridizations of *D. melanogaster* embryos were performed using FITC- and DIG-labelled RNA probes, a sheep anti-DIG primary antibody (Roche) and a mouse anti-FITC primary antibody (Roche). Embryos were mounted in 80% glycerol and photographed under differential interference contrast (DIC) optics.

2.3. Immunohistochemistry of embryos

Embryos were fixed and treated as described in Zúñiga et al. (2009). Primary antibody was polyclonal anti-phospho-Smad (phospho S423 + S425, Abcam, 1:50) and secondary antibody was anti-rabbit Alexa 488 (Jackson, 1:500). Nuclear staining was made with ToPro (Molecular Probes, 1:200). Fluorescently-labelled embryos were mounted in DAKO or in 3:1 Glycerol:PBS. Confocal images were collected using confocal microscope C2+ (Nikon) and processed using NIS-Elements Microscope Imaging Software (Nikon) and Image J (NIH).

2.4. RNA extraction and preparation of spike mRNAs

Total RNA from *nos*-Gal4>*lacZ*, *nos*-Gal4>UAS-*dpp* embryos (N = 100–200) at late stage 5 (cellularization) to early stage 7 (gastrulation) of development was extracted as described in Zúñiga et al. (2009). RNA was quantified using Qubit RNA HS Assay Kit (Thermo Fisher) and the integrity was assayed in Tape station 2200 (Agilent Technologies). For microarray experiments, 50 pg of spike mRNAs was added to each RNA preparation prior to labelling. The three spike genes were *Bacillus subtilis* tryptophan operon, *trpCDEF* (ATCC 87485), diaminopimelate decarboxylase gene *lysA* (ATCC 87482) and dihydrodipicolinate reductase gene, *dapB*, (*ATCC 87486*). Each vector consists of bacterial cDNA cloned into the *Xho*I and *Bam*HI sites of a modified pBluescript II-KS+ vector in which a poly(dA) stretch follows the *Bam*HI restriction site. From each vector, RNA transcripts containing a poly(A) tract were generated using the Riboprobe Combination System (Promega). Predicted transcript sizes were confirmed using denaturant agarose gel electrophoresis.

2.5. Probe synthesis and microarray hybridization

To prepare the fluorescent probes, total RNA was amplified using the Amino Allyl MessageAmp II aRNA Amplification Kit (Ambion, Texas) following the manufacturer's instructions. Labelled aRNA was purified using QIAquick columns (Qiagen), yield and specific activity of each probe was determined by absorption spectroscopy. Pairs of Cy3 and Cy5 labelled aRNA probes (2.5 μg/probe) were pooled, fragmented (Ambion RNA Fragmentation Reagent) and hybridized to the *D. melanogaster* microarrays. The experimental samples were tagged with Cy5 and the control samples with Cy3. In separate hybridizations, the labelling of the samples was dye-swapped.

Oligonucleotide *D. melanogaster* microarrays were purchased from Microarray Inc. and contained 14,593 probes designed from the Gadfly release 3.1 database, they represent 13,664 genes and 17,899 transcripts. Oligonucleotides corresponding to genes coding for the spikes RNAs were randomly distributed in different blocks throughout the array. Microarrays were pre-washed in 50 mL of pre-hybridization buffer (5 × SSC, 0.1% SDS, 0.1% BSA Fraction V) for 60 min at 42 °C, then 5 times in ddH_2O for 1 min at room temperature. After the pre-washing, microarrays were dried by centrifugation. Then, microarrays were hybridized with labelled probes in a hybridization solution containing 20% formamide, 5× SSC and 0.1% SDS. Hybridized slides were sequentially washed 4 times by 15 min in 2× SSC, 0.1% SDS, 4 times by 5 min in 0.1× SSC, 0.1% SDS and then rinsed 4 times by 1 min in 0.1× SSC.

2.6. Microarray experimental design and data analysis

Two independent control (*nos*-Gal4>*lacZ*) and experimental (*nos*-Gal4>UAS-*dpp*) samples (biological replicates) were hybridized onto nine slides, and dye-swap replicates were conducted in the first hybridization. Images were processed using the software ScanArray Express (Perkin Elmer) to align both channels at different PTM gain. Image quality was assessed by *q.com* descriptors included within the R function, (Wang et al., 2001). Additionally, control spots (spike controls, empty spots and random oligomers) were analysed separately to distinguished high quality hybridized slides. Data from slides were processed to