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Transcriptional analysis of the *dachsous* gene uncovers novel isoforms expressed during development in *Drosophila*

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ABSTRACT

The *Drosophila* cadherin-related protein Dachsous (Ds) plays a prominent role in planar cell polarity (PCP) and growth. The regulation of these two processes is based on the interaction between Ds and Fat proteins, generating an intracellular response required for tissue polarization and modulation of Hippo pathway activity. Here we have performed a comprehensive molecular study of the *ds* gene during larval development that has shown an unexpected complexity in its transcriptional regulation and revealed the expression of hitherto unsuspected transcripts. Also, knockdown of several isoforms provides new evidence on the importance of the cytoplasmic domain in the mechanism of action of Ds during development.

1. Introduction

Numerous tissues undergo an additional level of organization through an evolutionary conserved mechanism called planar cell polarity (PCP), which contributes to the development of fully functional organs of a precise size and shape. The *Drosophila* cadherin-related Dachsous (Ds) and Fat (Ft) proteins and their homologues in vertebrates participate in the control of PCP and growth (reviewed in [1]). The current model is based on the heterophilic interaction between these two single-pass transmembrane proteins. This interaction generates a signaling cascade mediated by the Ft intracellular domain, which results in cytoskeletal reorganization and the modulation of the Hippo pathway [2,3].

During *Drosophila* wing formation, Ds regulates several processes such as the orientation of cell division, the specification of proximal-distal (P/D) patterning, vein formation, and wing hair orientation [4–6]. Thus, herein the term PCP/patterning will be used to encompass all these PCP processes. In addition, Ds can control tissue growth, modulating the activity of the Hippo signaling pathway. This role of Ds is related to its ability to regulate the expression of genes such as *four jointed* (*fj*) [7], *dally* and *dally-like* (*dlp*), key modulators of the Hedgehog, Wnt and TGF-β signaling pathways involved in cell proliferation and patterning [8,9].

Significant progress has been made in understanding the mechanism by which Ft regulates PCP and growth, due to the identification of cytoplasmic partners that interact with specific sequence motifs of Ft cytoplasmic domain (CD) [10–13]. However, the molecular mechanism of Ds regulating PCP and the Hippo pathway is less well understood. Reported data provide genetic evidence that Ds can regulate these processes in two different ways: in a cell-autonomous or a non-cell-autonomous manner. Therefore, PCP and growth can be also regulated by Ds independently of Ft [7,8,14,15]. At present it is difficult to envision how the current model, based on the requirement of the extracellular domain of Ds, can explain the cell-autonomous function.

In this work, the molecular analysis of the *ds* gene reveals the expression of several *ds* transcriptional variants in imaginal discs and the brain during larval development. We have characterized three novel isoforms that encode the soluble proteins DsEx, Ds1, and DsIntra. Moreover, we show that the cytoplasmic isoforms can regulate not only PCP/patterning and growth but also

Author contributions: E.R.-Y. carried out the qRT-PCR and genetic experiments and analyzed the data. L.V. carried out RNA experiments, conducted the confocal imaging, and generated Ds antibody. J.S. carried out protein experiments and analyzed data from the sequencing and RACE assays. I.R. designed and supervised experiments, analyzed data, and wrote the paper. All authors discussed the results and contributed extensively to the work presented in this paper. All authors gave their final approval for publication.

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mitochondrial activity, indicating a relevant role of the cytoplasmic domain in the mechanism of action of Ds during development.

2. Materials and methods

2.1. Drosophila genetics

ds mutant alleles ds^{1}, 30AG4 and ds^{38k}, and the Gal4 lines 638-Gal4, en-Gal4, hh-Gal4 and tub-Gal4 were obtained from the Bloomington Stock Center. puc-lacZ (puc^{E69}; [16]). RNAi lines were obtained from VDRC (http://stockcenter.vdrc.at/control/main). ds^{1} and *30AG4* are weak alleles caused by transposable elements inserted in coding exons of the extracellular domain [17]. ds^{38k} is a spontaneous mutation that does not affect the coding exons (our sequencing analysis). In turn, ds^{36D} is a strong allele generated by the P-excision of ds^{05142} [5]. UAS-DsFL [18] comprises the complete coding region of full-length Ds [17]. UAS-ectoDs encodes a large extracellular domain anchored to the cell membrane [15]. The UAS-DsIntraV5 transgene was generated by fusion of the cDNA sequence (nt 10153 to 11464; Genbank: L08811.2) and one copy of the V5 epitope to the C-terminus (Ct) in the pUAS-T vector. The UAS-DsExGFP transgene comprises from exon2 to exon10 (nt 796–6832; Genbank: L08811.2) and one copy of the GFP sequence joined to the 3′ end of exon10. The aa sequences of the fusion are: UAS-DsIntraV5 ...GTRMSRGPFE**GKPIPN**... and UAS-DsExGFP ...LITTVGAGT**MVSKGE**... (ds sequences are underlined and V5 and GFP sequences appear in boldface). To analyze wing phenotypes, the transgenes dsRNAi-cyt, dsRNAi-ex, DsFL, UAS-ectoDs, DsExGFP and DsIntraV5 were overexpressed in a wild-type (wt) background under the control of *638G4*, *enG4* and *hhG4* drivers. For quantitation, the A-PCV and the wing area between 4 and 12 wings of each genotype were traced using Adobe Photoshop CS3. The average for distance and area was normalized to control females. Measurement of the A-PCV distance was carried out as described in [11]. The wing area parameter was calculated by dividing the posterior area (P) by the total area (T) (Fig. S1).

2.2. Statistical analysis

In all the experiments significance was determined using the Student's *t*-distribution (two tailed; two sample equal variances). A *P* value of <0.05 was considered statistically significant.

2.3. Immunohistochemistry

Wing imaginal discs were dissected and stained as described in [19]. Primary antibodies used for histology were: rabbit anti-Dachsous (Ds^{ex} 1:1000 [20], guinea pig anti-Dachsous (Ds^{cyt} 1:500; this study), mouse anti-COXIV (1:50; Invitrogen), rabbit anti-β-galactosidase (1:10000; Cappel), rabbit anti-Caspase 3 (1:200, Cell Signaling), mouse anti-Dlp (1:100; Developmental Studies Hybridoma Bank), and mouse anti-V5 (1:2000; Invitrogen). Proteins were visualized using fluorescent secondary antibodies (Jackson InmunoResearch Laboratories). The analysis of Ds expression in mitochondria was determined by co-immunostaining with COXIV (complex IV) in fat body cells of third instar larvae [21]. ROS production was evaluated in live tissues using Dihydroethidium (DHE). The *Drosophila* imaginal discs were dissected following the protocol described in [22]. The guinea pig Ds antibody against the cytoplasmic region was generated from a GST fusion protein (pGEX4T2 vector) containing the amino acids 3410–3700 (AAF51468.3). Imaginal discs were mounted in Vectashield (Vector Laboratories) and Mowiol. Images were acquired by using a Zeiss Confocal LSM510 microscope.

2.4. Quantitative real-time PCR and data analysis

Total RNA was extracted from three independent biological samples (imaginal discs and brains) of wt and *tubG4*/*UAS-dsRNAi* larvae to determine the expression of individual exons by two-step real-time PCR (RT-PCR). The first cDNA strand was synthesized with the Transcriptor First Strand cDNA Synthesis kit (Roche) using 2 μg of total RNA and oligo (dT) primer. For the second step of qRT-PCR amplification, a pair of primers for each coding exon was designed following the recommendations of the Universal Probe Library Assay Design Center (Roche Applied Science). The *Tbp* and *eIF-2α* genes were used as endogenous controls for the analysis of RNA quantity and quality. The primer sequences and amplicon size are indicated in Table S1. All PCR reactions were carried out in technical duplicates using HotStart Taq polymerase (Qiagen) and SYBR green (Qiagen) in an optical 384-well plate with ABI 7900HT (Applied BioSystems). For absolute quantification (standard curve method), the *pUAS-dsFL* (exons 2–12) construct was used as standard [18] (Table S2). For the standard curves, 5 serial 10-fold dilutions from the same reaction mixture of *pUAS-ds* were amplified. For relative quantification, the normalization of different biological samples was performed using the comparative Ct ($2^{-\Delta\Delta Ct}$) method after an efficiency calculation for each primer pair (Table S3). For statistical analysis of the experimental groups, differences in mean values were analyzed with the one-tailed non-parametric Student *t*-test. A *P*-value <0.05 was considered statistically significant. Data analysis was carried out using Integromics RealTime StatMiner software (http://www.integromics.com/StatMiner).

2.5. Analysis of RACE products

cDNA libraries were obtained with the SMARTer RACE (rapid amplification of cDNA ends) cDNA amplification kit (Clontech) from two independent biological samples of the total RNA of wt imaginal discs and brains. PCR amplification was performed with the Advantage 2 PCR kit (Clontech). Each RACE product was cloned in the pCRII vector (Invitrogene), and at least 5 independent clones were sequenced. To confirm the RACE results, RT-PCR products were amplified using the Expand High Fidelity PCR System (Roche). The location of primer sequences within the exons and the complete cDNA sequences of DsEx, Ds1 and DsIntra are shown in Fig. S2 (Genbank accesion numbers: KT935293, KT935292, KT935294).

2.6. Northern blot analysis

The total RNA was extracted from brains and imaginal discs of third instar larvae using TriPure Isolation Reagent (Roche). RNA was quantified in a nanodrop ND-1100. The quality of each sample was checked in an Agilent 2100 bioanalyzer. For Northern blot analysis, 20 μg of total RNA were loaded on 1% agarose-formaldehyde gels and run in MOPS1X buffer. After RNA transfer, the nylon membranes (Roche) were hybridized at 60 °C in a modified Church & Gilbert's hybridization buffer [23]. Detection was carried out with CDP-Star according to the DIG RNA Detection Protocol (Roche). The digoxigenin-labeled anti-sense RNA probes were synthesized according to the same protocol. As templates we used two different cDNA fragments: one of 5.2 kb (exons 2–11; clone 119 in Clark, 1995) and the 1.4 kb fragment of exon12 corresponding to the coding sequence of the cytoplasmic domain. The size of the bands was determined by extrapolation from a standard curve using RNA molecular size markers.

2.7. Western blot analysis

For Western blot analysis, total proteins were isolated from imaginal discs and brains in Ringer's solution supplemented with

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