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A combinatorial enhancer recognized by Mad, TCF and Brinker first activates then represses *dpp* expression in the posterior spiracles of *Drosophila*

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Abstract

A previous genetic analysis of a reporter gene carrying a 375-bp region from a *dpp* intron (dppMX-lacZ) revealed that the Wingless and Dpp pathways are required to activate *dpp* expression in posterior spiracle formation. Here we report that within the dppMX region there is an enhancer with binding sites for TCF and Mad that are essential for activating dppMX expression in posterior spiracles. There is also a binding site for Brinker likely employed to repress dppMX expression. This combinatorial enhancer may be the first identified with the ability to integrate temporally distinct positive (TCF and Mad) and negative (Brinker) inputs in the same cells. Cuticle studies on a unique *dpp* mutant lacking this enhancer showed that it is required for viability and that the Filzkorper are U-shaped rather than straight. Together with gene expression data from these mutants and from *brk* mutants, our results suggest that there are two rounds of Dpp signaling in posterior spiracle development. The first round is associated with dorsal–ventral patterning and is necessary for designating the posterior spiracle field. The second is governed by the combinatorial enhancer and begins during germ band retraction. The second round appears necessary for proper spiracle internal morphology and fusion with the remainder of the tracheal system. Intriguingly, several aspects of *dpp* posterior spiracle expression and function are similar to demonstrated roles for Wnt and BMP signaling in proximal–distal outgrowth of the mammalian embryonic lung.

Keywords: *Drosophila*; Dpp; Mad; TCF; Brinker; Combinatorial signaling; Posterior spiracle enhancer; Gene regulation

Introduction

Secreted proteins in the transforming growth factorβ (TGFβ) and Wingless/Int-1 (Wnt) families have important roles in many species. In *Drosophila*, the TGFβ family member *decapentaplegic* (*dpp*) influences numerous developmental events (e.g., Ashe et al., 2000; Waltzer and Bienz, 1999). Typically, the transcription factor Mad is responsible for Dpp-dependent gene expression (e.g., Massagué et al., 2005). The *Drosophila* Wnt family member *wingless* (*wg*) also influences many developmental decisions (e.g., Cordero et al., 2004; Hatini et al., 2005). In canonical Wg signal transduction, the transcription factor TCF is largely responsible for Wg-dependent gene expression (e.g., Willert and Jones, 2006).

In a genetic analysis, we demonstrated that combinatorial signaling by the Wg and Dpp pathways regulates *dpp* expression in the posterior dorsal ectoderm. At stage 11, the *dpp* intron-derived reporter gene dppMX-lacZ is expressed in two bilaterally symmetrical clusters of dorsal ectoderm cells in the eighth abdominal segment. At stage 17, dppMX expression is present in posterior regions of the tracheal system: (1) in posterior portions of the dorsal trunk branches that connect the

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anterior and posterior spiracles; (2) in the spiracular branches that connect the spiracular chambers to the dorsal trunk branches; and (3) in the spiracular chambers of the posterior spiracles (Takaesu et al., 2002a). Interestingly, the posterior spiracles are the only functional tracheal opening at hatching and for the first larval instar only the spiracular branches and the spiracular chambers participate in gas exchange (Manning and Krasnow, 1993).

Substantial genetic and fate map data show that the development of the posterior spiracles is separable from the remainder of the tracheal system (Martinez-Arias and Lawrence, 1985; Jurgens, 1987). Consistent with these studies, the spiracular branches and posterior spiracles are not detected with reagents commonly employed to study tracheal development such as *trachealess* or *breathless* (Takaesu et al., 2002a). In addition to *dpp*, gene expression studies revealed that the transcription factors *spalt*, *cut* and *esg* are also expressed in posterior spiracle cells. An analysis of their mutant phenotypes suggests that these genes are required for cell fate choices in the spiracles (Hu and Castelli-Gair, 1999; Merabet et al., 2005). Further, developmental studies of the external morphology of the posterior spiracles revealed a role for Rho signaling in invagination and formation of the spiracular lumen (Simões et al., 2006). Here we report that *dpp* posterior spiracle activity does not influence cell fate or external morphology but instead regulates spiracle internal morphology.

Viability and cuticle studies of a unique mutant we created showed that the MX intronic region of the *dpp* locus is required for posterior spiracle development but not for dorsal–ventral patterning. This result contrasts with the prevailing wisdom that considers all *dpp* posterior spiracle defects simply downstream consequences of dorsal–ventral patterning defects. Instead, our data suggest that there are two rounds of Dpp signaling in posterior spiracle development. The first round is necessary for setting up the posterior spiracle field in association with dorsal–ventral patterning of the blastoderm stage embryo. The second begins during germ band retraction, is regulated by an enhancer in the MX region and appears to regulate fusion of the posterior spiracles with the dorsal trunk branches late in embryogenesis. In addition, the enhancer within the dppMX region contains binding sites recognized by TCF and Mad that are essential for activating *dpp* expression. There is also a binding site recognized by Brinker that appears to be employed to repress dppMX expression late in development. To our knowledge, this enhancer is the first one known that provides cells with the ability to respond to sequential positive and negative signals from three transcription factors.

Materials and methods

Molecular biology

To create the *dpp*−ΔKX rescue construct, we began with a *Not*I to *Pst*I clone from the *dpp* chromosome walk (St. Johnston et al., 1990). This is a subclone from the 8-kb *Eco*RI fragment that constitutes the *dpp* rescue construct (Padgett et al., 1993). A 100-bp deletion from *Kas*I to *Xba*I (ΔKX) was made in the subclone. An *Sph*I to *Eco*RI subclone from the 8-kb *Eco*RI fragment was also generated. The *Sph*I to *Pst*I fragment of the *Sph*I to *Eco*RI subclone was replaced with the *Sph*I to *Pst*I fragment from the ΔKX version of the *Not*I to *Pst*I subclone. An *Xho*I (from the MCS) to *Sph*I fragment from the 8-kb *Eco*RI clone was then inserted upstream of the ΔKX version of the *Sph*I to *Pst*I fragment. The 8-kb *Eco*RI fragment was recreated minus the 100-bp *Kas*I to *Xba*I fragment and utilized to generate the *dpp*–ΔKX rescue construct in Casper4. To create the *dpp*−ΔKX reporter gene, a Bluescript clone of the dppMX reporter gene was digested with *Kas*I and *Xba*I, the ends were polished with T4 ligase and reclosed. To create the dppMX-MadM1+2 and dppMX-TCFM1+2 reporter genes, oligos bearing mutations that match those shown in Supplemental Table 1 were incorporated into a dppMX subclone with Stratagene's Quick-changeII kit (La Jolla, CA). Then each fragment was excised and inserted into the HZR-lacZ transformation vector as described (Takaesu et al., 2002a).

Drosophila genetics

PB{Gal4}43 is as described (Horn et al., 2003), PS{Gal4}8B4B is as described (Takaesu et al., 2002b), P{UAS-pan.TCF.ΔN}4, P{UAS-wg.H.T:HA1}6C and P{UAS.Brk}2.2 are as described (Flybase, 2007) and P{UAS.Dpp}5 is as described (Staehling-Hampton and Hoffmann, 1994). In Gal4-UAS crosses where a transgene was not homozygous, viable experimental embryos were positively identified by the absence of blue-balancer or GFP-balancer chromosomes. dpp^{Hin46}, dpp^{Hin47} and dpp^{Hin61} are haploinsufficient alleles as described by St. Johnston et al. (1990). The CyO.23 balancer carrying the *dpp* rescue construct is as described (Padgett et al., 1993). Strains homozygous for the *dpp* rescue or *dpp*−ΔKX rescue construct on chromosome III and a dpp^{Hin} allele on chromosome II over *In(2LR)Gla* were generated via standard schemes. Lethality tests of these strains were conducted as described (Hoffmann and Goodman, 1987). Cuticles were prepared as described (Wharton et al., 1993). brk^{F124} and brk^{M68} are null or nearly null allele as described (Jazwinska et al., 1999; Lammel et al., 2000; Saller et al., 2002). The P[lacW] insertions $esg^{B7\text{-}2\text{-}22}$ and brk^{37} are as described (Flybase, 2007).

Biochemistry

Expression of the histadine-tagged HMG box of TCF-A in pET15b (Novagen) was induced according to van de Wetering et al. (1997). Protein was purified using Ni^{2+}-coated resin (New England Biolabs). Oligos were labeled with [γ-^{32}P]ATP and purified by PAGE. Binding reactions were conducted according to Xu et al. (1998). Expression of the MH1 domain of Mad fused to GST in pGEX (Amersham) was induced according to Kim et al. (1997). Protein was purified with a GSTtrap column (Amersham). Oligos were end labeled with [γ-^{32}P]dCTP and purified with QIAquick Nucleotide Removal kit (QIAGEN). Binding reactions were conducted according to Kim et al. (1997). Expression of full-length Brk protein (Minami et al., 1999) was conducted with the TNT Rabbit Reticulocyte Coupled Transcription Translation System (Promega). Oligos were labeled and purified as described for Mad-MH1. Binding reactions were conducted according to Sivasankaran et al. (2000). Bound and unbound oligos were separated using 5% native PAGE in 0.5× TBE buffer followed by autoradiography. All oligo sequences are shown in Supplemental Table 1.

Developmental biology

mRNA in situ hybridization to embryos with a digoxigenin-labeled *dpp* cDNA was conducted as described (Takaesu et al., 2002a) and with a fluorescent-labeled cDNA as described (Kosman et al., 2004). Immunohistochemistry was performed as described (Johnson et al., 2003). The following primary antibodies were utilized: rabbit α-Spalt (Kuhnlein et al., 1994), rabbit α-phospho-Smad1 (Persson et al., 1998), rabbit α-lacZ (Organon Teknika) and mouse monoclonal 2B10 α-Cut (Jack et al., 1991). Secondary antibodies include biotinylated goat α-rabbit and α-mouse (Vector Laboratories), Alexa Fluor 488- and 633-conjugated goat α-rabbit and α-mouse and horseradish peroxidase-conjugated goat α-rabbit and α-mouse (Molecular Probes). The Vectastain Elite kit (Vector Laboratories) was employed to detect biotinylated antibodies and the TSA Amplification kit (Molecular Probes) was utilized to detect HRP-conjugated antibodies.